Syzygium aromaticum (L) Merril & Perry – ITS LEAF ESSENTIAL OIL NANOSPHERES ATTENUATES, RESCUES β AMYLOID PATHALOGY IN TRANSGENIC Drosophila *melanogaster* MODEL OF ALZHEIMER'S DISEASE - A ROAD TO THERAPEUTICS.

A dissertation submitted to

THE TAMIL NADU Dr.M.G.R.MEDICAL UNIVERSITY

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In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY

IN

BRANCH III-PHARMACOGNOSY

Submitted by

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Under the Guidance of

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MAY-2018

CERTIFICATE

This is to certify that the dissertation entitled " ***Syzygium aromaticum (L) Merril & Perry*– ITS LEAF ESSENTIAL OIL NANOSPHERES ATTENUATES, RESCUES β AMYLOID PATHALOGY IN TRANSGENIC *Drosophila melanogaster* MODEL OF ALZHEIMER'S DISEASE - A ROAD TO THERAPEUTICS."** is a bonafide work done by **Mrs.G.HEMALATHA (Reg. No: 261620702),** DEPARTMENT OF PHARMACOGNOSY, COLLEGE OF PHARMACY**, MADURAI MEDICAL COLLEGE, MADURAI-625020** in partial fulfilment of the Tamil Nadu Dr. M.G.R. Medical University rules and regulations for the award of **MASTER OF PHARMACY IN PHARMACOGNOSY** under my guidance and supervision during the academic year 2017-2018.

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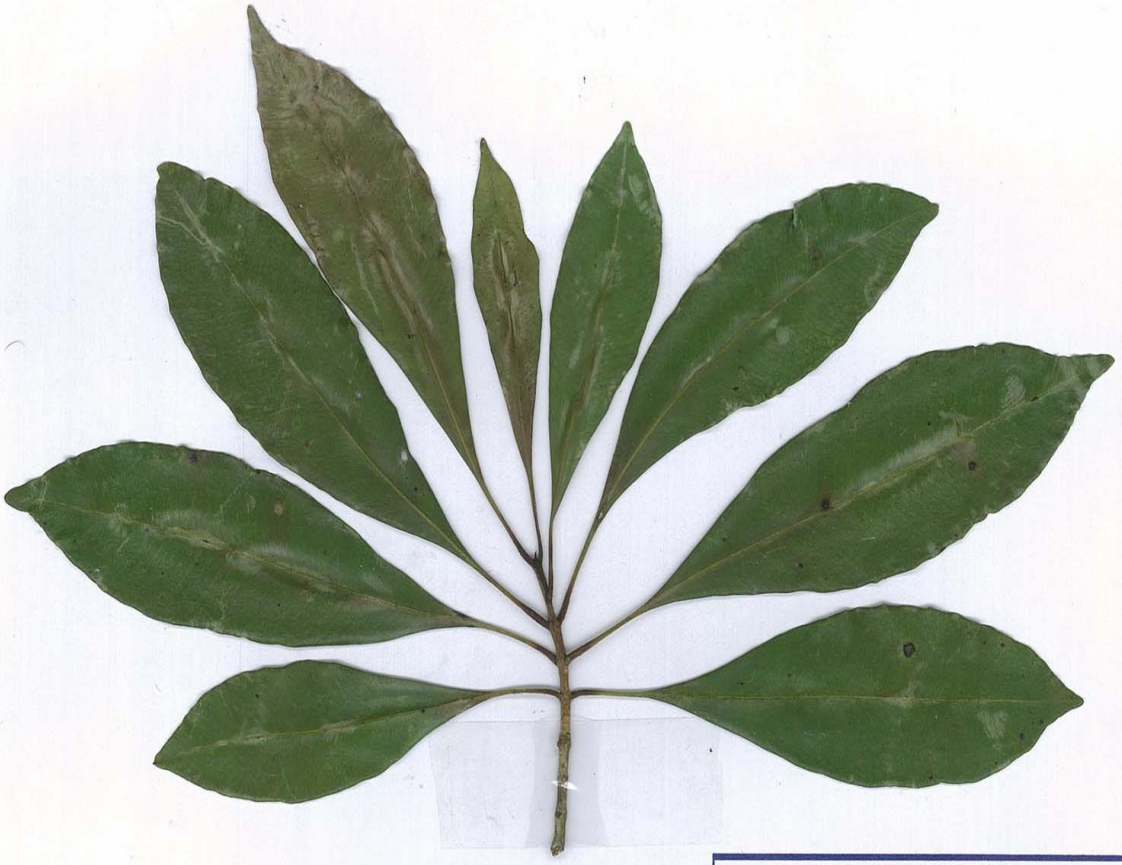

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NAME : SYZYGIUM AROMATICUM

FAMILY : MYRTACEAE

GENUS : SYZYGIUM

SPECIES : AROMATICUM

LOCALITY : HALIEY BERIYARESTATE
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DATE : 11.9.2017

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DEDICATED TO MY LOVABLE

FAMILY, GURU AND THE ALMIGHTY

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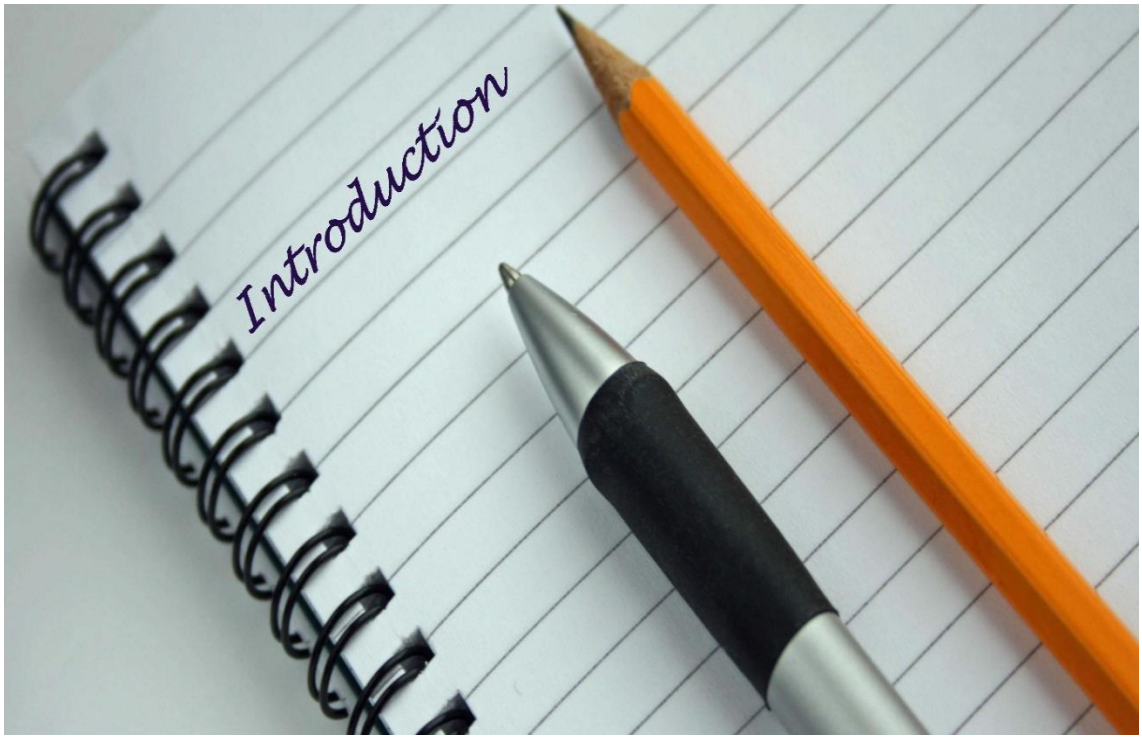


INTRODUCTION

INTRODUCTION

INDIA AND TRADITIONAL SYSTEM OF MEDICINE

The word 'Environment' has originated from French word "environ". It means surroundings which includes each and everything outside the plant and influences directly or indirectly the life of the plant. This constitutes an integral part of the earth's ecosystem. Each component of the environment is called environmental factor. Plants grow best within ranges of various factors like temperature, soil moisture, soil nutrients, light, air pollutants, humidity, soil structure and pH. Although these factors affect all plants are frequently grown or kept in cultural particles (fertilization, irrigation, spraying with pesticides) that may affect their growth considerably. According to the WHO, over 80% of the world's population relies on traditional forms of medicine, largely plant based to meet primary health care needs. In India, the collection and processing of herbal plants and its products contributes a major part each year to the national economy, as a source of both full and part time employment1.Studies suggested that a large number of those employed are women. In recognition of the significance of the sub-sector and the fact it is largely undocumented, the World Bank and the IDRC Medicinal plants Network (IMPN) agreed to produce this state of the art report on the herbal sector in India. The report suggests that despite a wealth of resources (biological, human and funds being available, due to the lack of a coordinated approach which considers sustainable and equitable development to be short as well as long term goals for the sub sector) has resulted in the simultaneous underutilization and overexploitation of the valuable plant resource. Plants are one of the most important sources of medicines. The application of plants as

medicines date back to prehistoric period. In India the references to the curative properties of some herbs in the Rig-Veda seems to be the earliest records of use of plants in medicines. The medicinal plants are extensively utilized throughout the world in two distinct areas of health management; traditional system of medicine and modern system of medicine. The traditional system of medicine mainly functions through two distinct streams (1) Local or folk or tribal stream and, (2) Codified and organized Indian system of medicines like Ayurveda Siddha and Unani etc. India is a well-known treasure of medicinal plants diversity, since ancient time as many plant species have medicinal value and are being used by local people for curing a variety of ailments. The traditional societies possess a rich knowledge of plants having various medicinal uses. The therapeutic uses of plant species have played an important role in the origin and evolution of many traditional herbal therapies in the developing countries especially in India. It is a well-known fact that the plants have been used as sources of medicine ever since the beginning of human civilization in the treatment of various diseases. Medicinal plant use as a source of herbal medicine are very much prevalent in the traditional health care system as a part of the cultural landscape of many developing countries. Herbal even today play an important role in rural areas with various locally produced drugs used as home remedies for various diseases. The traditional knowledge of medicinal plants has started disappearing with the passage of time due to scarcity of written documents and relatively low income or no income to the traditional herbal practitioners (Vaidya's). In the recent past the medicinal plants have regained a fair degree of recognition due to a growing faith in herbal medicines in view of their few or no side effects as compared to

modern system of medicine. However, at present this vast knowledge based information available on these precious plants is getting eroded due to changing socio economic and cultural values and illegal collections from the wild. Hence sincere efforts are to be made to document the uses of these herbs before they disappear. (Phondani P.C. 2011).

The concept of Ayurveda appeared and developed between 2500 and 500 BC in India. The literal meaning of Ayurveda is “science of life,” because ancient Indian system of health care aims on the man and his illness. In this the positive health means metabolically well-balanced human beings. According to Ayurveda, the disease evolves from the body due to external factors. Its vast literature in Sanskrit covering all aspect of diseases and therapeutics. The practice of Ayurveda therapeutics consisted of 8 sections divided into 180 chapters and listed 314 plants, which are used as medicines in India. As already discussed the Indian subcontinent is a vast repository of medicinal plants that are used in traditional treatment of diseases. Many researchers have long regarded the Indian systems of medicine as a rich source of knowledge. In India, around 20,000 medicinal plants have been recorded however traditional communities are using only 7,000 - 7,500 plants for curing different diseases. The medicinal plants are listed in various indigenous systems such as Siddha (600), Ayurveda (700) and Unani (700), Allopathic in which 30 plant species for ailments and majorities of the medicines are prepared from the plant and animal products, minerals and metals etc. Major pharmaceutical industries depend on the plant products for the preparation of Ayurveda medicines. The Ayurveda system of medicine is widely accepted and practiced not only in the Indian Peninsula but also in the

developed countries such as Europe, United States and Japan.Medicines derived from plants have been the first line of defence in maintaining health and combating diseases. In the 20th century, roughly 121 pharmaceutical products have been discovered based on the information obtained from the traditional medicine practitioners.

PLANTS IN TRADITIONAL MEDICINES

Ayurveda remains an important system of medicine and drug therapy in India. Plant alkaloids are the primary active ingredients of Ayurveda drugs. Nowadays the therapeutically active ingredients of many Ayurveda medicines are being identified and their usefulness in drug therapy being determined. But only a certain percentage of plants are used in traditional medicines. It is approximately estimated that of the identified 17,000 species, nearly 3,000 species are used in medicinal field. The indigenous systems of medicine in India are reported in Table 1 under supplementary material. The therapeutic properties of some Ayurvedic crude drugs support for their pharmacological claims. Detailed information about various formulations with respect to their methods of preparation as well as basic standards and are documented in Sărangdhara Samhita.

DRUG DEVELOPMENT FROM NATURAL SOURCES

Chemical entities from natural sources have become much simpler and have significantly contributed to the newer drug development from medicinal plants. Active biological compounds from natural sources have always been of great interest to researchers working on infectious diseases. Research to find out scientific evidence for claims of plants used for traditional system of

medicine has been intensified. In depth investigation on the chemistry and pharmacology of products originated from plants origin are much essential and this may lead to the discovery of drugs that can be used in the treatment of several disease. Further, these Ayurveda preparations are scientifically evaluated and disseminated properly, our population can be given better access to efficacious drug treatment and improved health status. Commercial exploitation of these plants frequently, degradation of natural resources are reported to be major threats to medicinal plants in India. Understanding of the plant knowledge used for herbal formulations can be extended for future scientific investigation near future.

MARKET POTENTIAL OF HERBAL MEDICINES

Herbal medicines continue to be a major market in U.S. pharmaceuticals and contribute a multi-billion dollar business. Approximately 1500 botanicals are sold as dietary supplements; formulations are not subject to Food and Drug Administration (FDA) clinical toxicity testing to assure their safety and efficacy. The Indian herbal drug market size is about $1 billion and the export of plant based crude drug is around $100 million. The current market potential of herbal medicine is estimated about $ 80-250 billion in Europe and USA. The current market size of the natural health products in China is about USD 650 million, of which imported herbal medicines account for USD 15 million. In response to the expected improvement in modern herbal medicine and reflective of their growing demand for natural medicines, 73 % of the respondents to a consumer survey indicated they would depend more on herbal medicine in the future (Perumalsamy R. 2008)

PLANT FAMILIES YIELDING IMPORTANT PHYTOCONSTITUENTS

Systematics has always been an important tool in pharmacognostical research and practice. Related families often contain similar types of constituents and an understanding of the systematic position of a medicinal plant species allows some deductions to be made about the secondary metabolites from the species. For e.g. many members of the mint family contain volatile oil. Review of literatures highlighted the pharmaceutically most important families.

Humans use species in most of the 450 angiosperm plant families. But a handful of these families are exceedingly vital for our existence. The diets of most cultures rely substantially on species of Poaceae and Fabaceae family. Solanaceae, Rosaceae, Euphorbiaceae, also provide many critical resources for humans and rank high in global importance. The family solanaceae represent one of the most economoically and medicinally important families of angiosperms.

NEURODEGENERATION (Wolozin.B. 2000)

Neurodegeneration is the shelter terminology for the progressive loss of structure or function of neurons, including death of neurons. Many diseases including Parkinson's, Alzheimer's, and Huntington's occur as a determination of neurodegenerative processes. As research progresses, many relationship appear which relate these diseases to one another on a sub-cellular level. Discovering these relationship offers hope for therapeutic advances that could mitigate many diseases simultaneously concurrently. There are many parallels correspondence between different neurodegenerative disorders including typical protein assemblies as well as

induced cell death. Neurodegeneration can be found originate in many different levels of neuronal circuitry ranging from molecular to systemic.

LINKS BETWEEN NEURODEGENERATIVE DISORDERS

GENETICS

Many neurodegenerative diseases are precipitate by genetic mutations, most of which are located in completely unrelated genes. In many of the different diseases, the mutated gene has collective feature individuality: a repeat of the CAG nucleotide triplet. CAG encodes for the amino acid glutamine. A repetition of CAG results in a polyglutamine (polyQ) tract. Diseases showing this are known as polyglutamine diseases.

POLYGLUTAMINE

A repetition in this causes dominant pathogenesis. Extra glutamine residues can achieve toxic properties through a variety of ways, including irregular protein folding and degradation pathways, corrected subcellular localization, and abnormal interactions with other cellular proteins. PolyQ studies often use a variety classification of animal models because there is such a clearly distinguish trigger – repeat expansion. Extensive research has been done using models of worms (*C. elegans*), fruit flies (*Drosophila*), mice, and non-human primates. It is important significant to note that mammalian data is often needed for FDA approval of drugs, so a bulk majority of the research is done using mice. Using data from the other animals (*C. elegans*and *Drosophila* primarily) is usually a precursor to finding the equivalent mammalian gene.

ALPHA-SYNUCLEIN

It can accumulate to form insoluble fibrils in pathological conditions characterized differentiate by Lewy bodies, such as Parkinson's disease, dementia with Lewy bodies, and multiple system atrophy. Alpha-synuclein is the fundamental structural component of Lewy body fibrils. In addition, an alpha-synuclein fragment, known as the non-A β component (NAC), is found originate in amyloid plaques in Alzheimer's disease.

INTRACELLULAR MECHANISMS -PROTEIN DEGRADATION PATHWAYS

Parkinson’s disease and Huntington’s disease are both late-onset and collaborated with the accumulation of intracellular toxic proteins. Diseases caused by the gathering of proteins are known as proteinopathies, and they are primarily caused by aggregates in the following structures:

- Cytosol, e.g. Parkinson's & Huntington's
- Nucleus, e.g. Spinocerebellar ataxia type 1 (SCA1)
- Endoplasmic reticulum (ER), (as seen with neuroserpin mutations that cause familial encephalopathy with neuroserpin inclusion bodies)
- Extracellularly excreted proteins, amyloid-β in Alzheimer’s disease There are two main avenues eukaryotic cells use to remove troublesome proteins or organelles.

UBIQUITIN–PROTEASOME

Protein ubiquitin along with enzymes is fundamental for the degradation of many proteins that cause proteinopathies including polyQ expansions and alpha-synucleins. Research indicates proteasome enzymes

may not be able to perfectly cleave these irregular proteins which could probably result in a more toxic species. This is the fundamental route cells use to degrade proteins.Decreased proteasome activity is persistent with models in which intracellular protein aggregates form. It is still unknown whether it or not these aggregates are a cause or a result of neurodegeneration.

AUTOPHAGY–LYSOSOME PATHWAYS

It form of programmed cell death (PCD), this becomes the favourable route when a protein is aggregate-prone meaning it is a poor proteasome substrate. This can be separate into two forms of autophagy, macro autophagy and chaperone-mediated autophagy (CMA).

MACROAUTOPHAGY

It is embarrassed with nutrient recycling of macromolecules under conditions of starvation, certain apoptotic pathways, and if lack, contribute to the formation of ubiquinated inclusions. Experiments in mice with neuronally confined macro autophagy-gene knock out strengthen intra neuronal aggregates leads to neurodegeneration.

CHAPERONE-MEDIATED AUTOPHAGY

It defects may also lead to neurodegeneration. Research has exposed that mutant proteins bind to the CMA-pathway receptors on lysosomal membrane and in doing so prevent their own degradation as well as the degradation of other substrates.

MITOCHONDRIAL DYSFUNCTION

The most characteristic form of cell death in neurodegeneration is through the intrinsic mitochondrial apoptotic pathway. This pathway determines the motivation of caspase-9 by regulating the release of cytochrome c from the mitochondrial intermembrane space (IMS). Reactive oxygen species (ROS) are normal by-products of mitochondrial respiratory chain activity. ROS concentration is intermediate by mitochondrial antioxidants such as manganese superoxide dismutase (SOD2) and glutathione peroxidase. Excessive production of ROS (oxidative stress) is a central feature of all neurodegenerative disorders. In addition to the generation of ROS, mitochondria are also embarrassed with life-sustaining functions including calcium homeostasis, PCD, mitochondrial fission and fusion, lipid concentration of the mitochondrial membranes, and the mitochondrial permeability transition. Mitochondrial disease leading to neurodegeneration is likely, at least on some level, to convolute all of these functions.

There is strong confirmation that mitochondrial dysfunction and oxidative stress participate a causal role appearance in neurodegenerative disease pathogenesis, including in four of the more well-known diseases Alzheimer's, Parkinson's, Huntington's, and Amyotrophic lateral sclerosis.

AXONAL TRANSPORT

Axonal swelling and spheroids have been observed recognized in many different neurodegenerative diseases. This represent that defective axons are not only present in diseased neurons, but also that they may cause certain pathological insult due to accumulation of organelles. Axonal transport

can be obstruct by a variety of mechanisms including damage to: kinesinandcytoplasmic dynein, microtubules, cargoes, and mitochondria. When axonal transport is severely disrupted a degenerative pathway known as Wallerian-like degeneration is often triggered.

PROGRAMMED CELL DEATH

Programmed cell death (PCD) is death of a cell in any form, mediated reconcile by an intracellular program. There are, however, connections in which these mediated pathways are artificially accelerated due to injury or disease.

APOPTOSIS (TYPE I)

Apoptosis is a manifestation of programmed cell death in multicellular organisms. It is one of the main particular types of programmed cell death (PCD) and incorporates a series of biochemical events leading to a characteristic cell morphology and death.

EXTRINSIC APOPTOTIC PATHWAYS

Occur when factors outside the cell stimulate cell surface death receptors (e.g. Fas) which result in the stimulation of caspases-8 or -10.

INTRINSIC APOPTOTIC PATHWAYS

Result from mitochondrial release of cytochrome c or endoplasmic reticulum malfunctions both of which contribute to the activation of caspase-9. The nucleus and Golgi apparatus are other organelles that have damage sensors which can lead the cells down apoptotic pathways.

PCD AND NEURODEGENERATION:

Current research, often in transgenic animal models, associate both apoptotic and non-apoptotic pathways in neurodegeneration. Different diseases may enter these pathways at various points, but once triggered can lead result to interdependent pathways of cell death. Generally, cell death in neurodegeneration is due to apoptosis and most frequently through the intrinsic mitochondrial pathway.

NEURODEGENERATION IN DIFFERENT DISORDERS

ALZHEIMER'S DISEASE

Alzheimer's disease is characterized by loss of neurons and synapses in the cerebral cortex and certain subcortical regions. This loss determines in gross atrophy of the affected regions, including with degeneration in the temporal lobe and parietal lobe, and parts of the frontal cortex and cingulate gyrus.

Alzheimer's disease has been describe as a protein misfolding disease (proteopathy), caused by aggregation of abnormally folded A-beta and tau proteins in the brain. Plaques are building up of small peptides, 39–43 amino acids in length, called beta-amyloid (also written as A-beta or Aβ). Beta-amyloid is a portion from a larger protein called amyloid precursor protein (APP), a transmembrane protein that introduce through the neuron's membrane. APP is critical to neuron growth, survival and post-injury repair. In Alzheimer's disease, an unknown process causes APP to be split into smaller fragments by enzymes through proteolysis. One of these fragments gives rise to fibrils of beta-amyloid, which form clumps that deposit outside neurons in dense formations known as senile plaques.

PARKINSON'S DISEASE

Parkinson's disease is a degenerative disorder of the central nervous system. It results from the death of dopamine-generating cells in the substantia nigra, a region of the midbrain; the cause of cell-death is unknown.

The mechanism by which the brain cells in Parkinson's are lost may consist of an abnormal accumulation of the protein alpha-synuclein bound to ubiquitin in the damaged cells. The alpha-synuclein-ubiquitin complex cannot be directed to the proteosome. This protein accumulation forms proteinaceous cytoplasmic inclusions called Lewy bodies. The latest research on pathogenesis of disease has shown that the death of dopaminergic neurons by alpha-synuclein is due to a defect in the machinery that transports proteins between two major cellular organelles-the endoplasmic reticulum (ER) and the Golgi apparatus. Some proteins like Rab1 may reverse this defect caused by alpha-synuclein in animal models.

Recent research suggests that impaired axonal transport of alpha-synuclein leads to its accumulation in the Lewy bodies. Experiments have revealed reduced transport rates of both wild-type and two familial Parkinson's disease-associated mutant alpha-synucleins through axons of cultured neurons.

HUNTINGTON'S DISEASE

HD causes astrogliosis and loss of medium intermediate spiny neurons. Areas of the brain are of compassionate corresponding to their structure and the types of neurons they contain, decrease in size as they cumulatively lose cells. The areas compassionate are mainly in the striatum, but also the frontal and temporal cortices. The striatum's subthalamic nuclei

send control signals to the globuspallidus, which initiates and modulates motion. The weaker signals from subthalamic nuclei thus cause decreased initiation and modulation of movement, leads in the characteristic movements of the disorder.

Mutant Huntingtin is an aggregate-prone protein. During the cells natural clearance process these proteins are retrogradely transported to the cell body for destruction by lysosomes. It is a possibility that these mutant protein aggregates damage the retrograde of important cargoes such as BDNF by damaging molecular motors as well as microtubules.

AGING AND NEURODEGENERATION

The greatest risk possibility for neurodegenerative diseases is aging. Mitochondrial DNA mutations as well as oxidative stress both lead to aging. Many of these diseases are late-onset, meaning there is some factor that modify as a person ages for each disease. One constant factor is that in each disease, neurons gradually lose function as the disease progresses with age.

Programmed cell death is ordinarily strictly regulated to ensure the integrity of tissues.

REASON FOR SELECTION OF THIS PLANT

It was reported that Myrtaceae family members have phytoconstituents with various pharmacological properties useful in the treatment of bacterial, fungal, infection. Sedative, digestive, diuretic, haemostatic, antidote for snake bite and insect stings, abscesses, wounds, skin infection, athlete’s foot in human, dermatophilosis in animals, reduce body heat, liver and spleen enlargement, . It was also claimed that these

plants merit detailed study which can prove useful in the discovery of lead compounds leading to novel and more efficacious drugs. (George R 2004, Dr.Mitra R 2007).

Syzygium aromaticum belonging to the family Myrtaceae commonly called as clove really do not have any match as a cheap natural and easily available plant.Cloves are the aromatic dried buds of a tree (*Eugenia caryophyllata* also sometimes *Syzgium aromaticum*) used as a spice in virtually all the world's cuisine. The term 'Clove' is derived from the French word 'Clou' and the English word 'Clout', both meaning 'nail'- from the likeliness of the flower bud of the Clove tree to a broad headed nail. Clove is known to possess antibacterial properties and is used in various dental creams, tooth pastes, mouth washes, and throat sprays to cleanse bacteria. It is also used to relive pain from sore gums and improves overall dental health. In dentistry, eugenol in combination with zinc oxide is used for temporary filling of cavities. Clove is an anodyne (an agent that soothes or relives pain) for dental emergencies. Cloves are aphrodisiac (an agent for arousing or increasing sexual desire or potency). Clove is used as an anti-inflammatory agent, due to its high content of flavonoids. Aroma therapists use pure clove oil to cure the symptoms of rheumatism and arthritis. Clove is used as a carminative, to increase hydrochloric acid in the stomach and to improve peristalsis. Apply the paste of clove powder in honey to treat acne. Paste of clove powder in water promotes faster healing of cuts and bites. Cloves can effectively cure many digestive problems. It is having medicinal qualities to cure flatulence, loose motions, indigestion and nausea. Cloves are useful in relieving the symptoms of diarrhoea, gastric irritability and vomiting.

Clove and clove oil boost the immune system by purifying the blood and help to fight against various diseases. Clove oil is effective in curing Athlete's foot and nail fungus. Cloves are good expectorants that promote the discharge of mucous and secretions in the respiratory passage. The aromatic clove oil, when inhaled can help soothe certain respiratory conditions like cold, cough, asthma, bronchitis, and sinusitis. It also helps in clearing the nasal tract. Cloves can effectively prevent the lung cancer as well as the skin cancer. Eugenol helps in minimizing the harmful effects of environmental wastes that can cause cancer of digestive system. Clove oil stimulates blood flow and circulation making it useful for the people having cold extremities. Cloves benefit the diabetic patients by controlling the blood glucose levels. Eugenol is powerful enough for preventing blood clots.

Sucking of a clove bud reduces desire for alcohol. Muscular cramps are often relieved, when the oil of clove is applied as a poultice near the affected area. Cloves also help prevent the breakdown in retina of the eye, which slows down macular degeneration and aids vision in the old age. The underlying mechanism is through the prevention of the breakdown of docosahexaenoic acid, which preserves vision in elderly people.

Researchers found that sniffing the spicy aroma of cloves reduces drowsiness, irritability and headaches. One drop of clove oil applied to the roof of the mouth can instantly relieve many headaches.

Clove enhances memory retention. It is recommended for relieving brain fog, lethargy and depressive state of mind. Research has shown that clove oil is an effective mosquito repellent. Clove may be looked upon as the champion of all the anti-oxidants known till date. The Oxygen Radical

Absorption Capacity test (ORAC) is a scale developed by U.S. Department of Agriculture for comparing antioxidantactivity. The ORAC score, of clove is over 10 million. A drop of clove oil is 400 times more powerful as an anti-oxidant than wolf berries or blueberries.

Review of literature showed lacunae exist in the pharmacognostic, phytochemical and pharmacological studies. The present study assesses the potential of *S aromaticum* in relation to its uses in epileptic seizure and in terms of findings on modern bio scientific research.

REVIEW OF LITERATURE

LITERATURE REVIEW

TAXONOMICAL CLASSIFICATION

(ITIS Report Taxonomic S.No 506167)

Kingdom	:	Plantae
Sub Kingdom	:	Viridiplantao
Intra Kingdom	:	Streptophyta
Super division	:	Empryophyta
Division	:	Tracheophyta
Subdivision	:	Spermatophytina
Class	:	Magnoliopsida
Super order	:	Rosanae
Order	:	Myrtales
Family	:	Myrtaceae
Genus	:	*Syzygium*
Species	:	*aromaticum*

VERNACULAR NAME

Common Name : Cloves, Carophyllus, Clovos, Caryophyllus

Botanical Names : *Eugenia caryophyllus*, *Syzygium aromaticum*

Names in Indian languages

Sanskrit : Bhadrasriya, Devakusuma, Devapuspa, Haricandana, Karampu, Lavanga, Lavangaka, Lavangam, Varala.

Hindi : Laung, Laumg, Lavang.

Malayalam	**:**	Grampu, Karampu, Karayampu.
Marathi	**:**	Luvang
Kannada	**:**	Lavanga, Daevakusuma, Krambu
Tamil	**:**	Kirampu, Ilavankam, Kiraambu, Kirambu,Grambu.
Telgu	**:**	Devakusumamu, Lavangamu, Lavangalu, Kaaravallu
Bengali	**:**	Lavanga.
Gujarati	**:**	Lavang
Punjabi	**:**	Laung
Oriya	**:**	Labanga
Urdu	**:**	Laung, Loung

(Parle M & Deepa K 2011)

SYNONYM(S) :

- *Caryophyllus aromaticus*
- *Eugenia caryophyllata*
- *Eugenia caryophyllus*

HISTORY

Archeologists have found cloves in a ceramic vessel in Syria, with evidence that dates the find to within a few years of 1721 BC. In the third century BC, a Chinese leader in the Han Dynasty required those who addressed him to chew cloves to freshen their breath. Cloves were traded by Muslim sailors and merchants during the Middle Ages in the profitable Indian Ocean trade, the clove trade is also mentioned by Ibn Battuta and even famous *Arabian*

Nights characters such as Sinbad the Sailor are known to have bought and sold cloves from India.

Until modern times, cloves grew only on a few islands in the Moluccas (historically called the Spice Islands), including Bacan, Makian, Moti, Ternate, and Tidore. In fact, the clove tree that experts believe is the oldest in the world, named *Afo*, is on Ternate. The tree is between 350 and 400 years old. Tourists are told that seedlings from this very tree were stolen by a Frenchman named Pierre Poivre in 1770, transferred to the Isle de France (Mauritius), and then later to Zanzibar, which was once the world's largest producer of cloves.

Until cloves were grown outside of the Maluku Islands, they were traded like oil, with an enforced limit on exportation.As the Dutch East India Company consolidated its control of the spice trade in the 17th century, they sought to gain a monopoly in cloves as they had in nutmeg. However, "unlike nutmeg and mace, which were limited to the minute Bandas, clove trees grew all over the Moluccas, and the trade in cloves was way beyond the limited policing powers of the corporation. (Parle M & Deepa K 2011)

ETHANO BOTANY DESCRIPTION

Syzygium aromaticum is a handsome evergreen tree that grows 30-40 feet high, with extensive, branching, yellowish bark, leaves that are 4 inches long and 2 inches wide, rose-colored flowers (the rose color is said to be exquisite), and berry-like fruit.

The Clove itself consists of a dark-brown, solid, nearly cylindrical calyx, somewhat tapering below, and above divided into four ovate lobes; these lobes clasp the four lighter-colored arched and imbricate petals, which form a

globular head and cover the numerous bent stamens.

The ovary is contained in the upper part of the adherent calyx, is divided into two cells, each containing about twenty ovules, and is crowned by a quadrangular disk, in the center of which the style is placed.

Cloves are ½ to 2/3 inches long and as obtained from different localities, vary somewhat in the shade of their brown color. The large, plump and deep-brown cloves, as obtained from the Moluccas, Zanzibar, etc., are preferred. The smaller, shrivelled and light-colored varieties, such as are often exported from Cayenne and the West Indies being considered inferior.

Cloves have a somewhat fatty appearance and a strong and highly aromatic odor, and a very pungent, warm and aromatic taste. A large number of oil-cells are observed in the petals and in the outer tissue of the calyx; the latter are placed in two or three irregular circles beneath the epidermis and yield some of their oil upon pressure with the fingernail.

Cloves partly deprived of their volatile oil are said to be occasionally used for adulteration; they are quite moist and usually without the heads. The stalks, which are more pungent than the Cloves themselves, are either sold honestly or are sometimes used to adulterate ground Cloves (National Dispensatory, 1887)

BOTANICAL DESCRIPTION

Syzygium aromatium tree is an evergreen tree, which grows to a height ranging from 8 – 12 m.Syzygium is a genus of flowering plant belongs to family myrtaceae. The genus about 1200 – 1800 species.Large square leaves and sanguine flowers in numerous groups of terminal clusters.The flower buds are at first of pale color and gradually become green, after which they develop

into bright red.Cloves are harvested when 1.5 – 2cm long and consist of a long calyx, terminating in four spreading sepals and four unopened petals, which form a small ball in the centre. (Parle M & Deepa K 2011)

GEOGRAPHICAL DISTRIBUTION

Clove is commercially cultivated in India, Madagascar, Sri Lanka, Indonesia and the south of China. Now-a-days it also cultivated in Bangladesh in a small scale. (Bhuiyan N I *et al* .,2010).

CLIMATE AND SOIL

Clove trees grow well in rich loamy soils of the humid tropics and can be grown successfully in the red soils of the midlands of Kerala as well as in the hilly terrain of Western Ghats at higher elevations in Tamil Nadu and Karnataka.

A cooler climate with well distributed rainfall is ideal for flowering; it thrives well in areas receiving an annual rainfall of 150-300 cm. The site selected for cultivation of clove needs good drainage, since crop cannot withstand water logging .(Parle M & Deepa K 2011).

PROPAGATION:

Seed Propagation : The seed has a short viability of about 2 weeks so should be sown as soon as it is ripe in shady nursery beds, placing the seeds about 25mm deep in the soil. About 70% of the seeds germinate, usually after 1 - 6 weeks]. Plant out when they are about 25cm tall. Cuttings of terminal leafy softwood, kept in a frame at high humidity until they have rooted.

HARVESTING AND PROCESSING

The trees begin to flower in 6 years. Full bearing is achieved by about 20 years and the production continues for 80 years or more. Bearing between years shows much variation.

Clove clusters are handpicked, when the buds reach full size and turn pink but before they open. At this stage, they are less than 2 cm long. They are spread thinly on mats and stirred frequently for uniform drying.

Well dried cloves will snap cleanly with a sharp click across the thumb nail and weigh about one third of the green weight. The opened flowers are not valued as a spice. Harvesting has to be done without damaging the branches, as it adversely affects the subsequent growth of the trees.

On an average, a clove tree yields 3.5-7.0 kg/year, depending upon the age, size and condition of the tree (Parle M & Deepa K 2011).

WHOLE PLANT:-

ETHANO MEDICINAL USES

- Clove (*Syzygium aromaticum)* is some commonly used spices in households worldwide.(Shailesh 2015)
- Clove is commonly used spices in the every Indian household for culinary purposes as well as in the treatment of various infections (Mittal M *et al.,*2014)
- *Syzygium aromaticum* has also been used as remedy in herbal medicine. (Dehghani F *et al.,*2012)
- It was reported that have been used in folk medicine as antimicrobial agents since ancient times .(Grayer, R.J. and J.B. Harborne, 1994, Cowan, M.M.,1999.)

- This plant has been used for centuries as food preservatives. (Mohammed KAK *et al.,*2016, Shan B *et al*.,2005**)**
- Cloves are used in Indian ayurvedic medicine and Chinese medicine and Western her balism and dentistry. (Khandelwal KJ *et al.,*2004)
- This aromatic plant used as a carminative, to increase hydrochloric acid in stomach and to improve peristalsis.(Wankhede TB 2015**,** Khandelwal KJ *et al.,*2004**)**
- In tropical Asia cloves have been given to treat such diverse infections as malaria, cholera and tuberculosis as well as scabies. (Bhowmik. D *et al.,*2012)
- Traditionally cloves have been usedto treat flatulence, nausea and vomiting.(Tiwari P 2014)
- Cloves are a natural anthelmintic. .(Ipsa Subhankari 2012, Nayak PL 2013 and James BP *et al*.,2000)
- *S. aromaticum* plant to be used as a local antiseptic and anesthetic. (Khandelwal KJ *et al.,*2004)
- Clove is an anodyne (an agent that relives pain) for dental emergencies. (Shan B *et al*.,2005)
- Applied to a cavity in a decayed tooth, it also relieves toothache. (Anderson L. *et al*.,2012)
- Clove is used anti inflammatory agent. (Shan B *et al*.,2005)
- Clove enhances memory retention. It is recommended for relieving brain Fog, lethargy and depressive state of mind. (Shan B *et al*.,2005)

PHYTOCHEMISTRY:

- Clove is one of the major vegetal sources of phenolic compounds such as flavonoids, *hidroxibenzoic* acids, hidroxicinamic acids and hidroxiphenyl propens. (Khalid A K M *et al*.,2016**)**
- Clove contain high content of Flavonoids. (Shan B *et al*.,2005)
- The main bioactive compound of clove Eugenol is, which is found in concentrations ranging from 9 3 81.70 to 14 650.00 mg per 100 g of fresh plant material (Khalid A K M *et al*.,2016**)**
- The phenolic acids and gallic acid are the compounds found in higher concentration. However, other gallic acid derivates as hidrolizable tannins are present in higher concentrations as compared with other compounds.(Shan B *et al* 2005)

PHARMACOLOGY

- It was reported that reduced levels of cytochrome P450 enzymes which specify the hepato-detoxification character of it. It also referred as vaso-relaxant effects. By considering all potent remedial properties, it also includes the anticancer properties.(Chowdhury M A R 2016).
- As medicinal plants mainly as antioxidant agents and has antimicrobial activities. (Shan B *et al*.,2005)

ANTIOXIDANT ACTIVITY

- The aqueous extracts of clove have more antioxidant effect tested by different *in vitro* methods as 2,2-diphenyl-1-picrylhydrazyl (DPPH); 2,2′-azino-bis (3-ethyl benzothiazoline-6-sulphonic acid) (ABTS),

oxygen radical absorbance capacity, ferric reducing antioxidant power, xanthine oxidase and 2-deoxiguanosine. (Dudonné S *et al*.,2009).

- The *in vitro* antioxidant activity analysis by the ABTS method showed the higher antioxidant activity related with the polyphenols content. (Shan B *et al*.,2005).

ANTIMICROBIAL ACTIVITIES:

- The antimicrobial activities of clove have been proved against several bacteria and fungal strains. It was reported the antimicrobial activity of different Indian spice plant clove(Sofia P K *et al.,*2007)
- The only sampled that showed complete bactericidal effect against all the food-borne pathogens tested *Escherichia coli* (*E. coli*), *Staphylococcus aureus a*nd *Bacillus cereus* was the aqueous extract of clove at 3%. At the concentration of 1% clove extract also showed good inhibitory action. (Sofia P K *et al.,*2007*)*
- Clove proved its enormous potential as food preservative among the other 30 plants analyzeddue to its antimicrobial aactivity(Dudonné S *et al*.,2009)
- Recently, many reports confirmed the antibacterial, antifungal, antiviral and anticarcinogenic properties of this plant. Clove in particular has attracted the attention due to the potent antioxidant and antimicrobial activities standing out among the other spices (Shan B *et al*.,2005)

LEAF

ETHANO MEDICAL USES :

- This aromatic plant leaves of clove used as a carminative, to increase hydrochloric acid in stomach and to improve peristalsis.(Wankhede TB 2015)
- The leaves are used for antiseptic and antipyretic (Chaveerach A *et al*., 2014).

PHARMACOGNOSY

- Leaves lanceolate, in pairs, acute at both ends 7.5 -12.3 cm , 2.5 - 3.75cm., gland-dotted, fragrant; (Anonymous 1976 Wealth of india
- These are simple, opposite ,coriaceous ,explitude, glbraous and aromatic.The Petiole is slender 2-3 cm long , swollen and pinkish at the base and with the leaf blade partly decurrent upon it in the upper portion.The lamina is lanceolate or narrowly elliptically or obovate , the apex is shortly or broadly acuminate and the base is cuneate. (Anonymous 1976).

LEAF OIL:

ETHNOMEDICAL USES:

- Traditionally clove leaf oil has been used to treat burns and cuts and even in dental care for alleviating toothache and infection.(Pulikottil S, Nath S 2015)
- Traditional uses of clove leaf oil include use in dental care as an antiseptic and analgesic.(Razafimamonjison G 2005.)

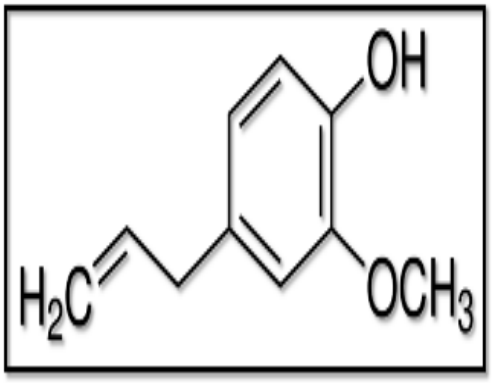
PHARMACOGNOSY:

- The yield of oil was 1.2% in 100 grams of clove leaves. (Indu S , Ambujam N M L 2010, Utpala P *et al*.,2014)
- It was reported that Clove leaves yield 3.0 – 4.8 % essential oil (Racing *et al*.,2001).

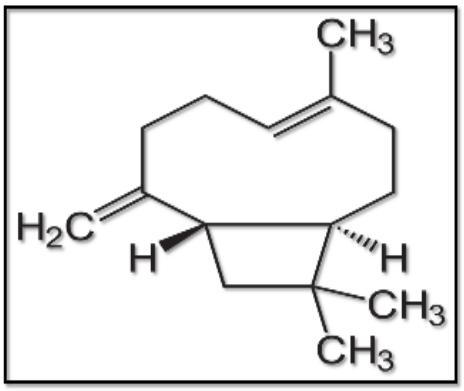
PHYTOCHEMISTRY

- It was reported that 38 components were identified in the leaf oil. The main components were eugenol (74.3%), eucalyptol (5.8%), caryophyllene (3.85%) and a-cadinol (2.43%) in leaf oil. (Bhuiyan N I *et al* .,2010).
- The oil contained 94.4% eugenol followed by B – caryophyllene (2.9%), nerol (0.79%) and B-caryophyllene oxide (0.67%) (Raina VK *et al* 2001).
- The leaf oil of clove from Madagascar contained 22 constituents, the chief constituents being eugenol (82.0%) and B-caryophyllene (13.0%). It contained higher level of B caryophyllene compared with bud oil (7.2%) (Sriviastava AS *et al*.,2005)
- A commercial sample of leaf oil obtained in Germany contained 76.8%. eugenol components. (Jirovetz L *et al.,* 2006)

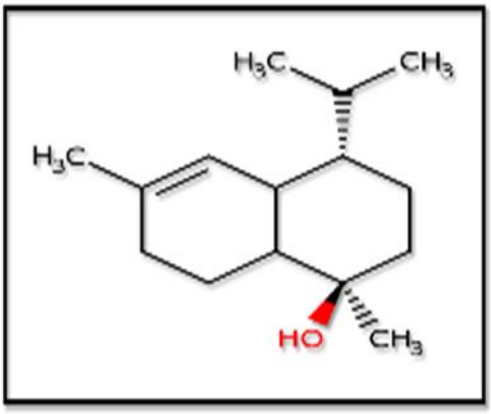
EUGENOL



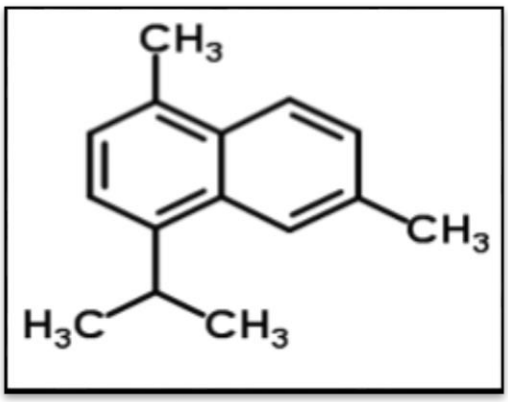
CARYOPHYLLINE



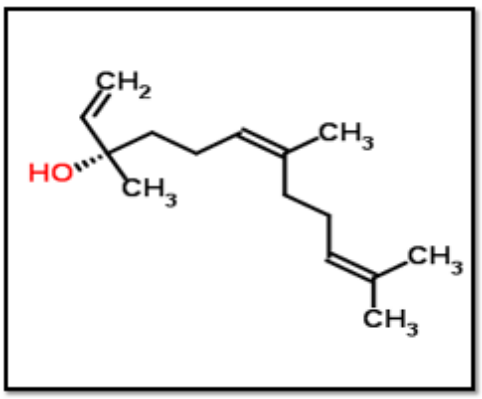
TRANSCALEDENE



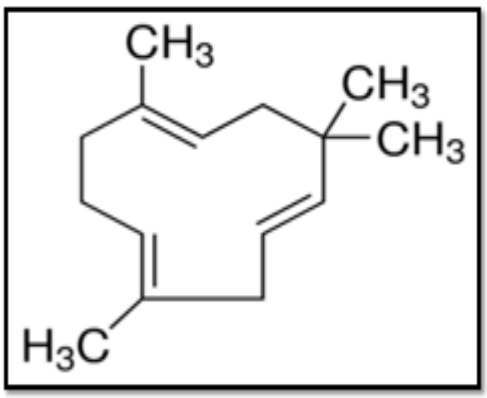
CADALENE



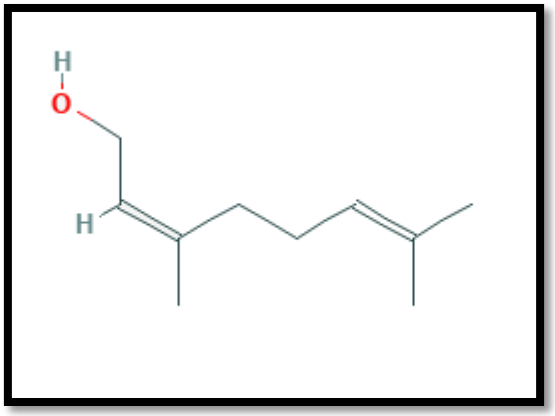
NEROLIDAL



HUMULENE



NEROL



- Eugenol reached its Highest percentage in

  (i) Expanded leaves = 84.00 – 90.48 %

  (ii)Mature leaves = 88.32 – 90.22 %

- Eugenyl Acetate reached its Highest percentage in

  Young leaves = 61.44 – 65.52 %

- Eugenol reached it Minimum percentage in

  (i) Young leaves = 25.43 – 30.38 %

  (ii)Expanded leaves = 58.29 – 61.53 %

- Clove leaf oil contain various classes of compounds. (Eg) Monoterpenes, Sesquiterpenes aldetrudes and kotones (Vernin G *et al*.,1994.)

- The yield of volatile oil was reported 3 – 4 %. It was observed that 38.3 % to 95.2%. Eugenol content variation in different development stage and at the sametime decrease of Eugenyacotic acid derivative and caryophylline. The various classes of constituted are alldehydes, ketones and some classes of are aldehydes, ketones and some classes of terpines. (Vernin G *et al*.,1994.)

PHARMACOLOGY

ANTIOXIDANT ACTIVITY

- It was reported that the essential oil of clove leaf possess scavenging activity against DPPH radical at concentrations lower than the concentrations of Eugenol, BHT and BHA. It also shows a significant inhibitory effect against hydroxyl radicals and act as an Iron chelator.(Gulein I *et al.,* 2004 , Jirovetz L *et al.*,2006)

BUD

ETHNO MEDICAL USES

- The clove has been used in India and China, for over 2,000 years, as a spice to check both tooth decay and counter halitosis (bad breath), mouth and throat inflammation. (Bhowmik D *et al*,2012)
- *S.aromaticum*, used most famously for toothache, and for mouth and throat inflammation.(Bhowmik. D *et al.,*2012)
- Cloves have historically been used in Indian cuisine (both North Indian and South Indian). In the north Indian cuisine, it is used in almost every sauce or side dish made, mostly ground up along with other spices. (Bhowmik D *et al.*,2012)
- Clove can be used to promote the flow of saliva and gastric juices. Clove can help to relieve the stomach pain or gas in the stomach and is used as an expectorant. (Bhowmik D *et al*,, 2012)
- Cloves were taken over the centuries for diarrhea, most liver, stomach and bowel ailments, and as a stimulant for the nerves (Bhowmik D *et al*,2012,)

- Traditionally cloves have been used to treat flatulence, nausea and vomiting .In tropical Asia cloves have been given to treat such diverse infections as malaria, cholera and tuberculosis, as well as scabies(Bhowmik D *et al* ., 2012)
- Taditional uses in America include treating worms, viruses, candida, various bacterial and protozoan infections (Bhowmik D *et al*,2012)
- Cloves became very popular as a medicinal flower, due to their ability to preserve foods, and mask the smell of poorly-kept foods**.(**Bullock and S. Harrison 2002)
- Clove is used to help digestion, prevent vomiting in pregnancy and has inhibitory effect on histamine production**.(**Abozid MM, El-Sayet SM 2013)
- The buds are used for symptomatic relief of toothache and inflammation-pain in the mouth and throat as they are sources of anti-microbial agents against oral bacteria commonly associated with dental caries and periodontal disease (Cai L et al 1996)
- This aromatic plant buds used as a carminative, to increase hydrochloric acid in stomach and to improve peristalsis.(Wankhede TB 2015)
- Along with the recreational uses of cloves, they are also said to be a natural anthelmintic. (Bhowmik D *et al*,2012)
- Spices and herbs were originally added for improving taste and also can naturally and safely improve shelf life of food products.(Maha M *et al.*,2012*).*

- Spices are also used for stabilizes in several food items from deterioration.(Jyothiprabha.V and Venkatachalam.P 2016)
- It was reported that Ayurvedic system of medicine, the clove is popularly known for its aphrodisiac property and used to treat male sexual disorders. (Sharma A 2011).
- It was reported that the buds of clove were used in folk medicine as diuretic,stomachic, tonic for cardiac muscles,aromatic condiment properties and condiment with carminative and stimulant activity several compounds(Bhuiyan MD et al 2010)

PHARMACOGNOSY

Color : Deep brown,

Odour : Powerful fragrant, intense fragrance

Taste : Warm, pungent, strongly sweet and slightly astringent, burning taste.

PHYTOCHEMISTRY

- It was reported that the presence of alkaloids in dichloromethane and aqueous extracts of clove(Shailesh,2015).
- Antioxidant study and quantification of phenolic compound of the extracts revealed that they have high antioxidant capacity. (Rahman Chowdhury M D A *et al.,*2016)
- Total phenolic content of (SAf) as 407.69 mg/g equivalent of gallic acid, indicate potent phenolic content (Rahman Chowdhury M D A *et al.,*2016).

PHARMACOLOGICAL ACTIVITY

- Clove extracts show high antifungal activity against *Rhizoctonia solani* (Lee S *et al*., 2003).
- Clove in particular has attracted the attention due to the potent antioxidant and antimicrobial activities standing out among the other spices(Diego FCR *et al.,*2014)
- Prolonged dose of *S.aromaticum* to swiss albino mice(8.35±2.07 and 8.77±1.71mmol/L) having ablity to reduce blood glucose level by compared with the standard drug of vildagliptin (50 mg/kg).Hence the methanolic extract of flower buds of *Syzygium aromaticum* (saf) could be used in managing oxidative stress in hyperglycemic condition. (Rahman Chowdhury M D A *et al.,*2016)
- *Syzygium aromaticum* acts as anti-fungal ,anti-inflammatory anti-microbial , immunomodulator ,anti-carcinogenic, anti-mutagenic.(Dehghani F *et al.,*2012)
- Antioxidant and estrogenic properties of *Syzygium aromaticum* may influence the sperm quality, sex hormones levels and the integrity of reproductive tissues. The present study was designed to verify the side effects of *Syzygium aromaticum*. Dehghani F *et al*.,2012)
- The high dose-treated animals showed a significant decline in sperm count, (250, 500 and 1000 mg/kg/day) motility and testosterone but a significant increase in estradiol concentration compared with the control group. The seminiferous tubules of extract-treated animals contained fewer sperms than in those of control animals. It seems that aphrodiastic

activity of the *S.aromaticum* extract, it reduced spermatogenesis. (Dehghani F *et al.,*2012).

- Clove offers neuroprotection against AlCl3-induced neurotoxicity.(Rami B Kassab, Amira A Bauomy, 2014)
- Acute administration of an ethanolic extract of clove enhances the learning and memory recall processes in mice which support the anti-oxidative, anti-amyloid-β peptide activity and cholinomimetic action of its eugenol component.(Rami B Kassab, Amira A Bauomy, 2014)
- Therapeutic properties of clove such as aphrodisiac, stomachic, carminative, antispasmodic, antiinflammatory,antioxidant, anti-hyperglycemic, anti-stress, antimutagenic,and allelopathic as well as antiseptic and anesthetic to relieve toothache among other pains. It is also reported to be useful in conceiving in high doses and acts as a contraceptive in low doses and useful in cataract few studies about the effect of clove on the neuronal activities.(Rami B Kassab, Amira A Bauomy, 2014)
- The clove has the ability to decrease the oxidative stress in diabetic rats ,it has ability to prevent diseases associated with oxidative stress.Moreover, eugenol and isoeugenol attenuated the levels of nitrite/nitrate, MDA and reactive oxygen species in acrylamide induced oxidative stress in rat's brain .(Rami B Kassab, Amira A Bauomy, 2014)

Hepato-protective activity

It was reported that the ethanolic extract of Clove showed the hepatoprotective activity on the paracetamol- induced liver injury. The extent of hepatic damage is assessed by the level of increased

cytoplasmic enzymes AST, ALT in circulation19 . Clove extract restored the activity of enzymes AST, ALT and ALP in serum towards normal values. These enzymes assess the functional status of the liver in both clinical and experimental settings. (Parle M & Deepa K 2011)

Anti-oxidant activity

- In DPPH free radical scavenging assay found IC50 values 13.204 µg/ml, total antioxidant of SAf as 356.5 mg/g equivalent of ascorbic acid. %reducing power capacity (SAf)of as 239.79±.075(Rahman Chowdhury M D A *et al.,*2016)
- Clove extract to diets already high in anti-inflammatory components (like cod liver oil, with its high ω-3 fatty acid content) brings a synergistic effect.(Parle M & Deepa K 2011)
- It was reported that Clove and Eugenol possess strong antioxidant activity than that of the synthetic antioxidant, BHA (butylated hydroxyl anisole) and Pyrogallol. (Parle M & Deepa K 2011)
- Clove has the highest capacity to give off hydrogen and reduce lipid peroxidation . With respect to the lipid peroxidation, the inhibitory activity of clove oil determined using a linolenic acid emulsion system indicated a higher antioxidant activity than the standard BHT (Butylated hydroxyl tolvene).(Parle M& Deepa K 2011)
- It also showed a significant inhibitory effect against hydroxyl radicals and act as an iron chelator . The metal chelating activity, bleomycin dependent DNA oxidation, diphenyl-p-picryl hydrazyl (DPPH) radical scavenging activity and the ferric reducing antioxidant power (FRAP) of different spices were measured in rat liver homogenate. Cloves showed

the highest DPPH radical scavenging activity & highest FRAP values. (Parle M & Deepa K 2011)

- The antioxidant activity of clove bud extract and its major aroma components, eugenol and eugenol acetate were comparable to that of the natural antioxidant α-tocopherol . Eugenol inhibited 5- lipoxygenase activity and leukotriene C-4 in human PMNL cells . (Parle M & Deepa K 2011)

Anti-platelet activity

It was found that both eugenol and acetyl eugenol, (two active constituents of clove) were more potent than aspirin in inhibiting platelet aggregation induced by arachidonate, adrenaline and collagen. In arachidonate induced-aggregation eugenol was at par with indomethacin. (Parle M & Deepa K 2011)

Anti-stress activity

It was reported that the clove extract reduced the development of cold restraint induced gastric ulcers and prevented the biochemical changes induced by sound stress such as elevated plasma levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphtase, glucose, cholesterol and corticosterone. Clove extract was also effective in increasing the latency of anoxic stress induced convulsions in mice. (Parle M & Deepa K 2011)

Insecticidal activity

It was reported that eugenol, isoeugenol and methyl eugenol show insecticidal activity to the storage pathogens of *Sitophilu szeamis* and

Tribolium costaneum. The clove leaf and bud oils showed potent insecticidal activity against the human head louse (*Pediculus capitis*). (Parle M & Deepa K 2011)

HEPATO – PROTECTIVE ACTION :

It was reported that ethanolic extract of clove demonstrated the hepato protective action on the paracotomol induced liver damage. The degree of hepatic harm is evaluated by the level of expanded cytoplasmic compounds. Clove concentrate restored the movement of compounds in serum towards ordinary qualities. (Parle M & Deepa K 2011)

BUD OIL:

ETHNOMEDICAL USES:

- Aroma therapists use pure clove oil to cure the symptoms of rheumatism and arthritis.(Shan B *et al*.,2005)
- The essential oil is used in aromatherapy .(Subhankari I and P.L. Nayak 2013)
- Clove buds yield approximately 15 to 20% of a volatile oil that is responsible for the characteristic aroma and flavor.**(**Shama I.Y *et al* 2013)
- clove essential oil was possibly used as food preservative.(Hamad A, Hartanti D 2015).
- Essential oil are used anodyne (painkiller) for dental purposes. (KJ Khandelwal *et al.,*2004)

PHYTOCHEMISTRY:

- It was reported that the Good quality clove buds contains 15-20% essential oil.(Gopalakrishnan *et al*.,1988)
- The volatile oil of cloves reported that it contain 85-92% eugenol. (Debjit Bhowmik *et al*,2012).
- It was reported that the oil from clove bud to contain 73.5-79.7% eugenol 4.5-10.7% Eugenol actate 7.3 – 12.4%. β-caryophyllene and 1.0 – 1.4%, humulene (Gaydou and Randriamiharison R 1987)
- It was reported that the oil from Madagascar was richer in eugenol (82.6%) and Eugenyl acetate (6%) compared with that of India (70 and 2.1% Respectively) Indian oil contained higher level of B – carryophyllene (19.5% against 7.2% in Madagascar oil) (Srivastava AS *et al*., 2005)
- There is 36 compounds of the volatile oil of clove buds were identified. The major components of the bud oil were eugenol (69.8%),β– caryophyllene (13%) and eugenyl acetate (16.1%) (Pino *et al.,*2001)
- Thirty one components were identified in bud oil with the main components being eugenol (49.7%), caryophyllene (18.9%), benzene,1-ethyl-3-nitro (11.1%) and3-(1-methylethyl) benzoic acid. (Bhuiyan N I *et al* .,2010).
- It was reported that clove buds from India contained 12.9 – 18.5% oil of which 44 – 55% was Eugenol, the pedicels contained 3 – 7.7% oil with 60 -72.4 % Eugenol. (Zachariah *et al* 2005).

- Wild uncultivated trees in Molucca yielded 3.0 -7.7 % bud oil. The oil contained no eugenol and quite different from the bud oil from cultivated trees. (Guenther,1950).
- The volatile oil of cloves reported that it contain 85-92% eugenol. (Bhowmik D *et al*,2012).
- Clove oil will stop the pain of a toothache when dropped into activity (Segacy EI.O *et al* 2007)
- Concentrations up to 18% of essential oil can be found in the clove flower buds.(Jirovetz L *et al*.,2006)
- Phenolic acids found in clove are the caffeic, ferulic, elagic and salicylic acids. Flavonoids as kaempferol, quercetin and its group (glycosilated) are also found in clove but in lower concentrations. 89% of the clove essential oil is eugenol and 5% to 15% is eugenol acetate and β-cariophylline (Jirovetz L *et al*.,2006)
- Another important compound found in the essential oil of clove in concentrations up to 2.1% is α-humulen.(Jirovetz L *et al*.,2006)
- Other volatile compounds present in lower concentrations in clove essential oil are β-pinene, limonene, farnesol, benzaldehyde, 2-heptanone and ethyl hexanoate. (Jirovetz L *et al*.,2006)
- The sesquiterpenos B – caryophyllene, B – caryophyllene epoxide, -humulene epoxide and eugenol (Zheng *et al*.,1992).
- The chemical composition of *S. aromaticum* was also investigated. Phenylpropanoids such as carvacrol, thymol and cinnamaldehyde flavonoid triglycosides eugenol, eugenol acetate, caryophyllene,

sesquetrepene ester were found in *S. aromaticum* extract. (Dehghani F,2012)

- The flower bud contains volatile oil (14% -21%), tannins(10% - 13%), phenol, sesquiterpenes, esters and alcohol. The most important constituent of clove is the phenylpropene, eugenol which gives this spice its pungent, distinctive aroma. .(Rami B Kassab, Amira A Bauomy, 2014)
- Eugenol makes up 70 % to 90 % of the essential oil and 15 % of the dry weight of clove buds.(Rami B Kassab, Amira A Bauomy, 2014)

PHARMACOLOGICAL ACTIVITY:

- One of the main constituents of clove oil (eugenol) exhibits broad antimicrobial activities against both Gram-positive, Gram-negative and acid-fact bacteria, as well as fungi . (Debjit Bhowmik *et al*,2012)
- The volatile oil of cloves reported that was highly active against a range of test microorganisms, being classified as bactericidal in nature. (Debjit Bhowmik *et al*,2012)

Anti-inflammatory activity

It was reported that Eugenol, the primary component of clove's volatile oils, functions as an anti-inflammatory agent. Clove also contains a variety of flavonoids, including kaempferol, rhamnetin and β-caryophyllene which also contributed to clove's anti-inflammatory and antioxidant properties26 . The essential oil of *Eugenia caryophyllata* had an anti-inflammatory effect matching to that of etodolac at 0.025 and 0.1 ml/kg

and to that of indomethacin at 0.05 and 0.2 ml/kg doses. (Milind Pand Deepa K 2011)

Mosquito repellent

It was reported that Clove oil gave the longest duration of 100% repellency (2-4 h) against three species of mosquitoes i.e. Aedes aegypti, Culex quinquefasciatus and Anopheles dirus under laboratory conditions using human subjects. (Milind Pand Deepa K 2011)

- The oil also inhibits the growth of *Fusarium verticilloides* (Velutie*t al*., 2004).

Anti -viral activity

- It was reported that Clove is a potent antiviral agent. Eugenin isolated from clove buds showed antiviral activity against Herpes Simplex virus at a concentration of 10 µg /ml16 . (Milind Pand Deepa K 2011)
- Essential oils from clove and eugenol showed various degree of inhibition, against *A.niger, S. cerevisiae* / mycoderma SP., *Lactobacillus acidophilus* and *B. cereus* as Estimated by the paper disc agar diffusion method.(Meena and sethi 1994).
- Clove oil and eugenol are reported to possess significant antifungal activity against ryebread spoilage fungi (Suhr and Nielsen 2003).
- Antiviral activity:Eugeniin from *S.aromaticum* tested against herpes virus strains being effective at 5 µg/mL, and it was deducted that one of the major targets of eugeniin is the viral DNA synthesis by the inhibition of the viral DNA polymeras (kurokawa M *et al.,*1998)

- clove oil showed potent anti-carcinogenic activity by inducing the detoxifying enzyme, glutathione –S– transferase in mouse liver and small intestine. (Zheng *et al*.,1992).

Anti-microbial activity

- Clove oil was found to be very effective agains*Staphylococcus species and* fungi, *Aspergillus niger* was found to be highly sensitive to the clove oil. (Milind Pand Deepa K 2011) Clove oil was found to be more effective than sodium propionate (standard food preservative) against some food borne microbes. (Milind Pand Deepa K 2011)
- Essential oil of clove, dispersed (0.4% v/v) in a concentrated sugar solution, had a germicidal effect against various bacteria *(S. Aureus, Klebsiella Pneumoniae*, *Pseudomonas aeruginosa, Clostridium perfringens, E.coli)* and *Candida albicans*. (Parle M & Deepa K 2011)
- Clove is also used for elimination of parasites from the digestive system. It has been found that a 0.05% solution of eugenol is sufficient to kill *Bacillus tuberculosis*. (Parle M & Deepa K 2011)
- Clove oil showed antimicrobial activity against some human pathogenic bacteria resistant to certain antibiotics . (Parle M & Deepa K 2011)
- It also shows activity against clinically relevant fungi including fluconazole- resistant strains15. (Parle M & Deepa K 2011)

Anti-pyretic effect:

- Eugenol was more effective in reducing fever than acetaminophen. . (Parle M& Deepa K 2011)
- It was reported that eugenol, the chief constituent of clove oil, showed marked antipyretic activity when given intravenously, intragastrically and centrally to rabbits made febrile by interleukin-1. It reduced fever primarily through a central action similar to that of common antipyretic drugs, such as acetaminophen. (Parle M & Deepa K 2011)

Anaesthetic effect :

- It was reported that Clove oil is found to be an alternative to Tricaine or MS- 222 the only registered anaesthetic for several fish speciesand also used as crab anaesthetic,Clove oil at the concentration of 100mg/l induced anesthesia within 1min30 in channel catfish (*Ictalurus punctatus*) .
- Clove oil and eugenol were reported to be acceptable anaesthetics for rabbit fish(Saiganus lineatus), coral reef fish (*Pomacentrus amboinensis*) and rainbow trout (*Oncorhynchus mykiss*)for use in aqua culture and aquatic research .
- Clove oil proved to be highly effective and easy to use on juvenile *Valamagugil cunnesius and Monodactylus argenteus*) tropical marine fish at the dose of 0.05ml/l. This dose anaesthetized the fish in less than a minute. (Parle M & Deepa K 2011)

STEM

PHARMACOGNOSY

- Main stems erect. 100cm in girth, often forking at a height of 1.5 – 1.8m. (Anonymous,1976)

STEM OIL:

PHARMACOGNOSY:

- The yield of 5.5-7 % of volatile oil from stem has less pleasant odour than oil from buds (Anonymous,1976).
- Clove stem yields 6% volatile oil (Gopalakrishnan*et N et al*.1988)

  The oil is a pale yellow liquid (Gaydou EM and Harisoa R 1987)

PHYTOCHEMISTRY:

- The oil generally contains a higher percentage of the free eugenol than the flower bud oils and only a small amount of eugenol acetate.(Anonymous,1976).
- Stem oil from Madagascar contains 77.10% Eugenol and 11.20% B caryophyllene as the major compounds (Gaydou and Harisoa R 1987)
- Stem oil used mainly in flavouring and perfumery and also to adulterate bud oil. .(Anonymous,1976).

SEED

PHARMACOGNOSY

- Oblong, soft, grooved on one side, 1.5cm long. Seeds will not germinate if dried out fresh seeds should be planted soon after removal from from the fruit.

ROOT OIL:

PHARMACOGNOSY:

- It is obtained by steam distillation of the roots of clove tree.
- The yield of oil from roots about 6 %,
- Color: Bright yellow

PHYTOCHEMISTRY:

The oil of clove root contains 85-95%eugenol

EUGENOL:

- Eugenol, a methoxyphenol component of clove (*Syzygium aromaticum*, Family *Myrtaceae*), has been reported for a number of pharmacological effects, including the antioxidant, antiinflammatory, analgesic, anesthetic, antipyretic, antiplatelet, antianaphylactic, antidepressant, anticonvulsant, antihyperglycemic, antibacterial, antifungal and antiviral effects. The uses and benefits of eugenol isolated from various sources including *Syzygium aromaticum* and *Ocimum sanctum* are many.
- Eugenol and its derivatives in clove are potent antioxidants, which could be due to their ability to form complexes with reduced metals; eugenol and structurally related compounds have been reported to inhibit iron-mediated lipid peroxidation and autooxidation of Fe^{2+} ion. Recently the effect of eugenol on hepatic glucose production and AMP-activated kinase signaling in hepatocyte and C57BL/6J mice suggested eugenol or eugenol-containing fractions as promising therapeutic agent. In the study, eugenol effectively ameliorated hyperglycemia through inhibition of hepatic gluconeogenesis by modulating calcium

calmodulin dependent kinase kinase-AMP activated kinase-CREB binding protein signaling pathway.

- In the liver, eugenol has been investigated for its antioxidant, antiinflammatory and DNA protective properties The dried flower bud of *Syzygium aromaticum* is used in food for aroma, and in medicine for its carminative, antispasmodic, anticarcinogenic and other properties. It is known to inhibit platelet aggregation and alter arachidonic acid metabolism in human platelet, besides showing the antiviral activity against herpes simplex, and antioxidant action in aflatoxicosis. The essential oil extracted from clove is used as topical application to relieve pain and promote healing.
- Clove is a rich source of free eugenol, eugenol acetate, caryophyllene, sesquetrepene ester, phenylpropanoid and β-caryophyllene,besides tannins and triterpenoids. It is used topically as analgesic in dental clinic and is antinociceptive, which might be due to α_2-adrenergic and opioidergic receptors, but not serotonergic receptor. Eugenol or 4-allyl-2-methyoxyphenol (molecular weight: 164.20), the major constituent in clove, is an allyl chain-substituted guaiacol (2-methoxyphenol) which can also lower uric acid and is indicated in the treatment of rheumatoid arthritis.
- It can induce apoptosis via the caspase-dependent pathway in human osteosarcoma cell. Eugenol is also known for its dose-dependent suppressive and enhancing effects on the immune response. Clove oil and eugenol microemulsions have been found beneficial in fatty liver and dyslipidemia. The formulation in microemulsion provides a

delivery system for oral administration of eugenol in homogeneous, water-based and thermodynamically stable dosage form. In CCl_4-induced hepatotoxicity in rats, eugenol protects liver injury when given concurrently or soon after the CCl_4 treatment. (Ali .S *et al*., 2014)

- Dried flower bud of *Syzygium aromaticum* (clove) is rich in eugenol, an antioxidant and antiinflammatory compound that can protect liver against injury. Clove, besides eugenol, also contains other pharmacologically active phytochemicals such as β-sitosterol and ascorbic acid. This study reports the effect of eugenol-rich fraction (ERF) of clove on liver cirrhosis induced by thioacetamide.(Ali. S *et al.,* 2014)

Drosophila Melanogaster AS MODEL ORGANISM

This study reported that Drosophila has provided a powerful genetic system in which to elucidate fundamental cellular pathways, developing and functioning nervous system. Now a days, Drosophila has been applied toward elucidating mechanisms of human neurodegenerative disease, including Alzheimer's, Parkinson's and Huntington's diseases. Drosophila genome, underscore the contributions that Drosophila as a model genetic systems t and s to contribute toward the understanding of human neurodegenerative disease. (Chan H and Bonini NM, 2000).

Drosophila MODEL ORGANISM FOR HUMAN NEURODEGENERATIVE DISEASE

In this study reported that *Drosophila melanogaster* as a important model system in the study of human neurodegeneration. These studies offer

reliable models for Alzheimer's, Parkinson's, and motor neuron diseases, as well as models for trinucleotide repeat expansion diseases, including ataxias and Huntington's disease, several signalling pathways including phosphatidylinositol 3-kinase (PI3K)/Akt and target of rapamycin (TOR), c-Jun N-terminal kinase (JNK) and bone morphogenetic protein (BMP) signalling, have been shown to be deregulated in models of proteinopathies, suggesting that 2 or more initiating events may trigger disease formation in an age-related manner. Moreover, these studies also demonstrate that the fruit fly can be used to screen chemical compounds for their potential to prevent or ameliorate the disease, can directly guide clinical research and the development of novel therapeutic strategies for the treatment of human neurodegenerative diseases. (Hirth F 2010).

ALZHEIMER'S DISEASE

In this study reported that Alzheimer's disease (AD) is most common degenerative brain diseases, affecting 11% of over 65 years of age and 50% over the age of 85 population. Alzheimer's disease (AD) disease brain tissue displays several unique pathological including senile neuritic plaques and neurofibrillary tangles. Senile neuritic plaques are extracellular deposits consisting of b-amyloid peptides, while neurofibrillary tangles are cytoplasmic aggregates composed of the paired helical filaments of hyper phosphorylated.These abnormal deposits form primarily in brain regions that are essential for cognition and memory, with Alzheimer's disease (AD) patients typified by dementia. . (Chan H and Bonini NM, 2000)

MODEL ORGANISM FOR ALZHEIMER DISEASE

In this study was suggested that Most of the genes implicated in AD pathogenesis have clear fly homologs. There is a fly homolog of APP (APP-like or APPL). Flies deficient for APPL exhibit a behavioural abnormality that can be rescued by a human APP transgene, indicating functional conservation between APPL and human APP. It is possible that other proteases may provide a similar function, as it has been reported that overexpression of human APP causes axonal transport defects and increases cell death in the larval brain in an Aβ-dependent manner. Some one study showed that Aβ peptide can be generated in *Drosophila* from a modified human APP transgene**.** (Lu B and Vogel H., 2009).

PARKINSON'S DISEASE

In this study reported that Parkinson's Disease (PD) is the most common movement disorder and the second most common neurodegenerative disease. The classical form of the disease is clinically characterized by muscle rigidity, resting tremor, bradykinesia, and postural instability. The movement abnormality in Parkinson's Disease (PD) is largely attributable to the deficiency of brain dopamine content, which is caused by degeneration of dopaminergic neurons in the substantia nigra. The most common forms of PD are sporadic with no known cause.(Lu B and Vogel H., 2009).

MODELING PARKINSON'S DISEASE IN *Drosophila*

In this study showed α-Syn in *Drosophila* has reproduced key features of Parkinson's Disease (PD), including Lewy body–like aggregate formation,

selective degeneration of dopaminergic neurons, and loco motor behaviour abnormality as detected by climbing ability in the negative geotaxis response. The fact that both mutant and wild-type α-Syn form aggregates in fly neurons and cause disease phenotypes supports the notion that improper disposal of aggregation-prone abnormal proteins can result in neurodegeneration. This is also consistent with the finding that genomic triplication of α-Syn in humans can cause Parkinson's disease (PD). Important two prominent features of *parkin*-mutant flies are mitochondrial pathology and apoptotic muscle degeneration. These mutant flies' exhibit sterility, reduced lifespan, reduced cell number and size, and hypersensitivity to oxidative stress. **(**Lu B and Vogel H., 2009).

IDENTIFYING SPOTTED WING *Drosophila*

In this study reported that Adult Spotted Wing Drosophila are 2-3 mm long, have rounded abdomens. Flies are light yellow or brown with red eyes. Dark unbroken bands are seen across the abdominal segments. Distinguishing characteristic is that the adult male Spotted

Wing Drosophila (SWD) has one distinctive dot on each of its wings along the 1st vein. Male Spotted Wing Drosophila (SWD) also have two dark bands on each of the forelegs. In these bands are known as combs and contain three to six teeth. Female Spotted Wing Drosophila (SWD) are harder to identify, as they do not have these wing spots. Female Spotted Wing Drosophila (SWD) have serrated ovipositors with two rows of serration that are longer than other vinegar fly species. **(**Timmeren SV, O'Donnell K *et al*.,2012)

HUNTINGTON DISEASE

In this study reported that Huntington disease (HD) is caused by a polyglutamine expansion mutation in huntingtin (HTT) that makes the protein toxic and aggregate-prone. The subcellular localisation of huntingtin and many of its interactors suggest a role in endocytosis, and recently it has been shown that huntingtin interacts indirectly with the early endosomal protein Rab5 through HAP40. (Ravikumar B, Imarisio S *et al*., 2008).

AIM AND OBJECTIVE

AIM AND OBJECTIVE

Volatile oils (V.O) are valuable natural products find applications in many area, including pharmaceuticals, cosmetics, perfumes, aromatherapy, phytotheraphy, spices etc. Attention of many scientists was attracted towards the screening of plants to study the biological activities of their oils from phytochemical and pharmacological to therapeutic aspects. This may be hopefully lead to new directions on plant applications and new perspectives on the potential therapeutic use of these natural products. VO are complex mixtures comprising many single compounds. The knowledge of its composition permits for a better and specially directed application.

Essential oils consist of monoterpenes and sesquiterpenes which are the lipophilic secondary metabolites of plants Various organic components are identified when essential oils are analyzed with a GC chromatograph which are as follows Terpene hydrocarbons (Monoterpene hydrocarbon, sesquiterpines), Oxygenated compounds (phenols, Alcohol,) Aldehyde, Ketones, Esters, Lactones, Coumarins, Ethers

ALZHEIMER'S DISEASE

Alzheimer's disease patient's exhibits marked decline in cognitive functions and severe behavioral abnormalities such as irritability, aphasia, apraxia, agnosia and restlessness. Neuritic plaques a core of β-amyloid aggregates covered by dead neurons, microglia and apolipoprotein E and neurofibrillary tangles are the major pathological lesions and neuronal death of an Alzheimer brain.

Dementia is failing memory and other intellectual function with little or on no disturbance in consciousness.β-amyloidal (Aβ) deposition is pathognenic for Alzheimer's disease (AD), but may occur in normal elderly people without apparent cognitive effect. Episodic memory impairment is an early and prominent sign of AD, but its relationship with Aβ burden in non-demented persons and in AD patient is unclear.

Cognitive deficits have long been recognized as severe and consistent neurological disorders associated with numerous psychiatric and neurodegenerative disease, such as AD, senile dementia, Parkinson's disease, Huntington's disease, korsakoff's syndrome, down's syndrome, pick's disease, trauma, chronic insomnia, epileptic disorder and attention deficit disorders.

The personality distortions interfere with the patient's professional life, social activities and relationships. Nootropic agent such as piracetam, aniracetam and choline esterase inhibitors like Donepezil® are being primarily used to improve memory, mood and behavior, but the resulting side effects associated with these agents have made their use limited.

NEED FOR THE STUDY

AD is a progressive brain disorder that gradually destroys a person's memory and ability to learn, reason, make judgment, communicate and carry out daily activities and ultimately leads to death**.** They are the most common form of dementia, a group of conditions that all gradually destroy brain cells and lead to progressive decline in mental function. The prevalence of mental and cognitive disorders, referred to as cognitive deficits which impinge on daily life and are thus of consequence to both those affected and society

doubles every five years between the ages of 60 and 95. Epidemiological studies have revealed that between two-thirds and Alzheimer's disease entirely or significantly causes three quarters of all cognitive deficits. Modulation of brain aging with complex extracts containing active photochemical has been useful in the aging of wild type rodents with encouraging results. Ayurvedic medicinal plants had successfully attenuated memory dysfunction induced by scopolamine, ethanol and diazepam. Recent advancements in the treatment of Alzheimer's disease and controlled studies have demonstrated that cholinesterase inhibitors delay cognitive decline by 6-10 months. Three AchE- inhibitors agents are available in Switzerland: tacrine, Donepezil and Rivastigmine. Tacrine is first generation AchE-inhibitors. Donepezil and Rivastigmine are second generation AChE-inhibitors, which are selectively centrally active. Despite of their relatively selective and central action, second generation AchE- inhibitors may still cause dose dependent cholinergic adverse effects. Thus the treatment of Alzheimer's disease has become a demanding interdisciplinary undertaking.Normal aging is known to deteriorate memory in human beings. Oxygen free radicals, the harmful by products of oxidative metabolism are known to cause organic damage to the living system, which may be responsible for the development of AD in elderly. The present study has been designed to investigate the nootropic potential of VO, a major phytochemical present in various speciesof the Myrtaceae family.

Encapsulation of bioactive compounds represents a feasible and efficient approach to modulate drug release, increase the physical stability of the active substances, protect them from the interactions with the

environment, decrease their volatility, enhance their bioactivity, reduce toxicity, and A significantly large part of current literature on the encapsulation of EOs deals with micrometric size capsules, which are used for the protection of the active compounds against environmental factors (e.g., oxygen, light, moisture, and pH),to decrease oil volatility and to transform the oil into a powder. Encapsulation in nanometric particles is an alternative for overcoming these problems but additionally, due to the subcellular size, may increase the cellular absorption mechanisms and increasing bio efficacy improve patient compliance and convenience.

Eos have promising potentials for maintaining and promoting health, as well as preventing and potentially treating some diseases. However, the generally low water solubility and stability, together with the high volatility and side effects associated with their use have limited their application in medicine. Nanotechnology is an innovative approach that has potential applications in medicinal and health research. Indeed, nanoparticles are a very attractive tool and are able to solve the major inconvenience of EOs use increasing the chemical stability in the presence of air, light, moisture and, high temperatures, factors which can lead to the rapid evaporation and to the degradation of the active components. In addition, nanocarriers ensure the easier and safer handling of the liquid substances by changing them in solid powders, determining retention of volatile ingredients and taste masking, setting up controlled release and/or consecutive delivery of multiple active ingredients, reducing toxic side effects, improving water solubility of hydrophobic ingredients, and enhancing bioavailability and efficacy.

AIM:

To study the pharmacognostic, preliminary phytochemical screening of the leaves *of Syzygium aromaticum* (L.) Merril & Perry Fam: *Myrtaceae* and also to investigate the *in vivo* neuroprotective effects of the nanospheres of the leaf volatile oil ***(***SALVONS) on β amyloid induced neuro degeneration in transgenic *Drosophila.*

OBJECTIVE

The objective of the study was divided into three parts.

PART 1: PHARMACOGNOSTICAL STUDY

- ❖ Collection and authentication of plant
- ❖ Morphological study of the plant
- ❖ Microscopy of the leaf
  - ➢ Anatomical study using light microscope
  - ➢ SEM analysis
  - ➢ Powder microscopy
  - ➢ Microscopic schedules
- ❖ Physio-chemical parameters
  - ➢ Ash values
  - ➢ Loss on drying
  - ➢ Extractive values

PART 2: PRELIMINARY PHYTOCHEMICAL SCREENING

- ❖ Qualitative analysis of the leaves for the presence of various phyto constituents
- ❖ Determination of flavonoid content, total phenolic content of the leaf,

- ❖ Determination trace elements by SEM
- ❖ Isolation of volatile oil from the leaves (VOSAL)
- ❖ Physico- chemical analysis of the isolated oil
- ❖ To study the GC-MS profile of the isolated VO
- ❖ Preparation of nano spheres of the VO (SALVONS)
- ❖ Characterisation of the prepared nanospheres

PART 3: PHARMACOLOGICAL STUDIES:

The 3R's ethical principle (**R**eduction, **R**efinement, and **R**eplacement) was implemented that help to minimize harms to vertebrate animals used in science.

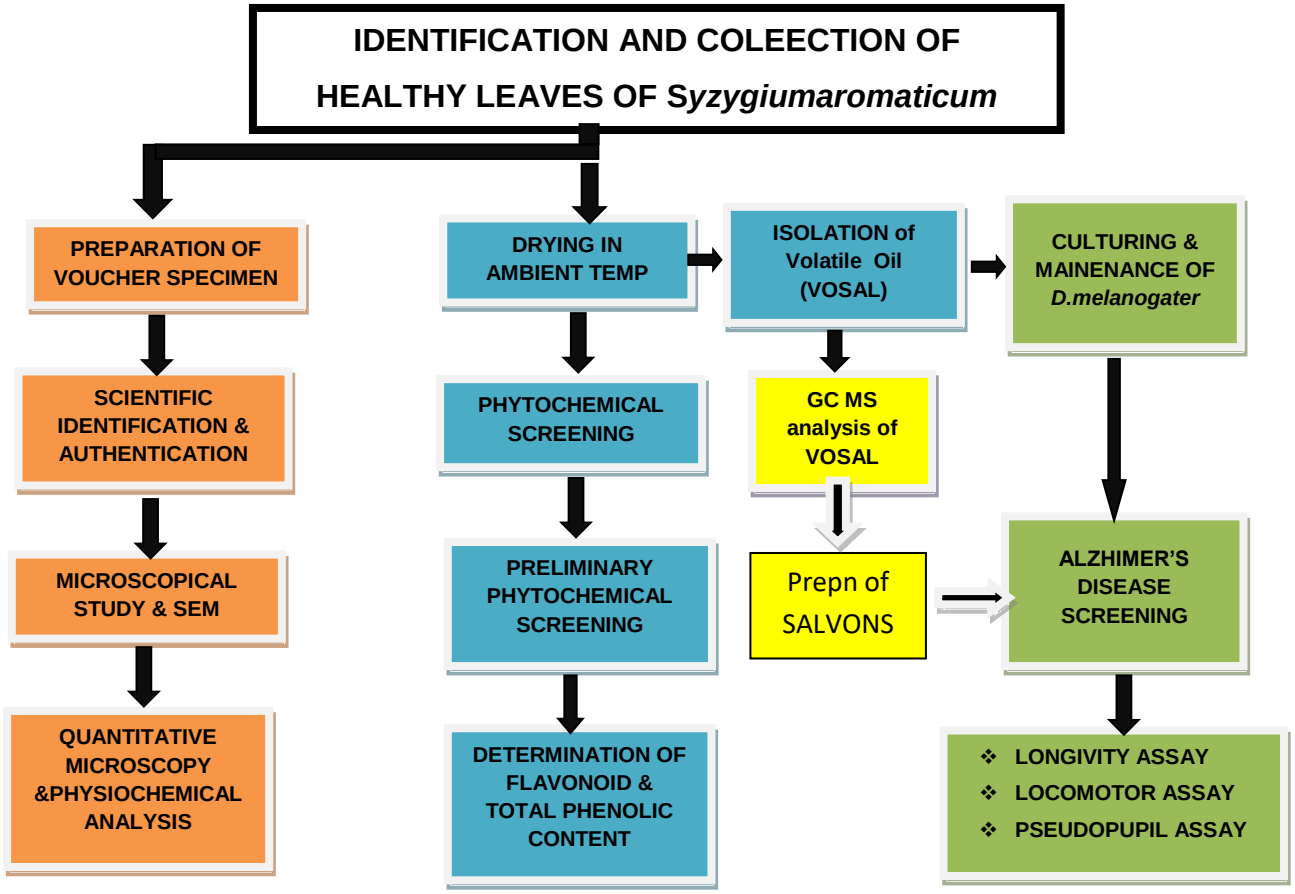
- ❖ Acute toxicological study using brine shrimp lethality bioassay(BSLA)
  - Production of *Artemia* nauplii
  - Cytotoxicity bioassay
  - Lethality concentration determination
- ❖ Effect on Mutagenesis of VOSAL on *Drosophila melanogaster In vivo* effect of the SALVONS ofon the transgenic *Drosophila melanogaster* with Aβ 42 induced neuro degeneration
- ❖ Preparation of medium for cultivation of *Drosophilla melanogaster.*
- ❖ Cultivation of normal and transgenic *Drosophilla melanogaster* model that over express human Aβ42 resulted in reduced lifespan, reduced locomotor activity and eye degeneration
- ❖ Effect of SALVONS on longevity of Aβ42 expressing *Drosophila*
- ❖ Effect of SALVONS on locomotor function by climbing assay of Aβ42 expressing *Drosophila*

- To perform Pseudopupil assay to determine rescued neuro degeneration in ommatidia of Aβ42 expressing *Drosophila*
- To examine the external morphology of the *Drosophila* eyes by nail polish imprint technique to examine any disruption in the ordered arrays of the ommatidia
- To study the morphology of the *Drosophilas*eye using SEM to examine any disruption in the ordered arrays of the ommatidia

MATERIALS AND METHOD

MATERIALS AND METHODS

RESEARCH DESIGN



MATERIALS AND METHODS

4.1. PLANT COLLECTION AND AUTHENTICATION:

Leaves of the plant ***Syzygiumaromaticum***selected for our study was collected from **Halieyberiyar estate, Idukki District**, Kerala, India during the month of July 2017 and was authenticated by **Dr.Stephen,** Department of Botany, American college, Madurai and **Dr.Sasikala,** Director (Retd) of Siddha Central Research Institute, Arumbakkam, Chennai.

LEAF DRYING AND PULVERIZING:

The leaves were collected and shade dried. It was powdered in a mixer. The powder was sieved in a No.60 sieve and kept in a well closed container in a dry place.

4.2. PHARMACOGNOSTICAL STUDIES:

Morphological and micro morphological examination and characterization of medicinal plants have always been accorded due credentials in the pharmacognostical studies. Botanical identity of the plants is an essential prerequisite for undertaking the analysis of medicinal properties of any plant. A researcher may succeed in getting a new compound or may find many useful pharmacological active properties in the plant. If the botanical identity of the plant happens to be dubious or erratic, the entire work on the plant becomes invalid. Thus it is needless to stress the botanical identity of the crude drug is the threshold in the processes of pharmacological investigations. The researchers should be equipped with all possible diagnostic parameters of the plant on which the researchers plan to work.

4.2.1. MORPHOLOGICAL STUDIES OF *Syzygium aromaticum*

Aerial part, leaf and petiole, flower, fruits and root were studied individually for its morphological characters by organoleptic test.

4.2.2. MICROSCOPICAL STUDIES ON THE LEAF OF *Syzygiumaromaticum*

COLLECTION OF SPECIMEN:

Care was taken to select healthy plants and for normal organs. Leaf, Petiole specimens were collected from a healthy plant by making a cut with petioles.The materials were cut into pieces and immediately immersed in fixative fluid FAA (Formalin – 5ml + Acetic acid – 5ml +70% Ethyl alcohol – 90ml).

DEHYDRATION:

After 24 hours of fixing , the specimens were dehydrated with graded series of ethyl alcohol and tertiary-butyl alcohol (Sass, 1940). The specimen is kept is in each grade of the fluid for about 6 hrs.Every time the fluid is decanted and immediately the specimen were flooded with next grade of fluid.

INFILTRATION WITH PARAFFIN WAX:

After dehydration, the shavings of paraffin wax were added to the vial containing the plant material with pure TBA. The paraffin shavings are added every 30mts at about 40-45ºC four or five times. Then the vials were filled with wax without damaging the tissues. The vial filled with wax is kept open in warm condition to evaporate all TBA, leaving the specimen in pure molten wax. The specimen filled with pure molten wax for 2 or 3 times by decanting the old wax every time.

CASTING TO MOLD:

A boat made out of chart board, by folding the margin, is used to prepare a mold of wax containing specimens. The paraffin along with the leaf and petiole specimen was poured into the boat. With the help of heated needles, the specimen were arranged in parallel rows with enough space in between the specimens. The block was then immersed in chilled water and allowed to cool for few hours.

SECTIONING:

The paraffin embedded specimens were sectioned with the help of microtome**.** The thickness of the sections was 10-12µm. Dewaxing of the sections was by customary procedure. The sections were stained with **Toluidine blue** as per the method published by O'Brien *et al* (1964). Since toluidine blue is a poly chromatic strain, the straining results were remarkably good and some **cytochemical reactions** were also obtained. The dye rendered pink colour to the **cellulose** walls, blue to the lignified cells, dark green to **suberin**, violet to the **mucilage**, blue to the **protein** bodies etc. Where ever necessary sections were also stained with **safranin** and **fast-green** and potassium iodide (for starch). For studying the stomatal morphology, venation patttern and trichome distribution, **paradermal sections** (sections taken parallel to the surface of leaf as well as **clearing** of leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid (Sass, 1940) were prepared. Glycerin mounted temporary preparations were made for macerated/cleared materials. Powdered materials of differentparts were cleared with sodium hydroxide and

mounted in glycerin medium after staining. Different cell components were studied and measured.

PHOTOMICROGRAPHS:

Microscopic descriptions of tissues are supplemented with micrographs wherever necessary. Photographs of different magnifications were taken.with Nikon labphot 2 Microscopic unit. For normal observations bright field was used. For the study of crystals, starch grains and lignified cells, polarized light were employed. Since these structures have birefringent property, under polarized light they appear bright against dark background. Magnifigations of the figures are indicated by the scalebars. (Johansen DA, 1940, Purvis MJ *et al*., 1966).

4.2.3. MICROSCOPICAL STUDY OF LEAF USING SCANNING ELECTRON MICROSCOPE:

SCANNING ELECTRON MICROSCOPE (SEM):

Movement of beam of foccussed electrons across an object forms a 3D image on a cathode - ray tube in a Scanning Eectron Microscope and it reads both the electrons scattered by the object and the secondary electrons produced by it. The electromagnetic lenses are used in SEM and focussing is done by the current. On photographic plate of screen the image is projected which gives comprehensive, quasi 3-D representation of the objects gives the ultra structure of plant cells. In addition , shows the unsuspected details and any undescribed characters. In other words the micrograph from SEM, shows the best possible structural details of the specimens. (Robards, 1970)

USAGE:

SEM info was handled as conventional character (or) character complexes as “pure” information without being broken down (or) interpreted as individual character using computer processing. The SEM information can be used some what at the superficial level just described to assist in solving taxonomic problem by confirming, changing (or) other grounds. It is also used often as diagnostic feature to avoid misleading by over simplified descriptions and one may find new kinds of microstructures not previously recognised and apprently simple structures may be extremely complex. Remarkably, poor conventional descriptions enabling taxonomic process of reducing a complex pattern to a few simple characters **(**Heywood VH, 1971).SEM plays a vital role when a specimen need to be satisfactorily defined in terms of characters. For most biological materials, maximum information is obtained by employing light and electron microscopy jointly and an attempt was made by applying SEM to the leaf of *S.torvum*, to pinpoint the positions of specific characters with in the cell, which can be easily seen in final image.

SEM SAMPLE PREPARATION:

Sample for SEM analysis were mounted on the specimen stub using carbon adhesive sheet. Small sample were mounted with I sq. cm glass slide and kept in carbon adhesive sheet.. Samples were coated with gold to a thickness of 100 AO using hitachi vacuum evaporator. Coated sample were analysed in a Hitachi Scanning electron Microscope 3000 H model.

4.2.4. POWDER MICROSCOPY:

MACERATION TECHNIQUE:

Maceration is the process of separation of individual cells by selectively dissolving the pectic middle lamella between the cells. The middle lamella binds the cells with each other forming different tissues. The middle lamella is dissolved by employing a chemical that dissolves the lamella to free the cells to obtain their three dimensional view.

MACERATION FLUID:

Jaffrey's maceration fluid is one that is commonly used for maceration (Johnsen DA, 1940). The fluid consists of equal volumes of 5% chromic acid and 5% nitric acid. The plant material is cut into small pieces and immersed in the maceration fluid. The fluid with the materials is kept at 55ºC for 3-5 hrs. Then the material is washed thoroughly with water and placed on a glass slide in a drop of Safranin (0.5%) for 15-20 min. The stain is drained carefully and mounted with a drop of dilute glycerine. The cells are spread well with a needle and the material is covered with cover slip. The slide so prepared is examined under the microscope to study different components of the macerate.

4.2.5. MICROSCOPIC SCHEDULES: (Wallis TE. 1953, Wallis TE, 1965, Iyengar MA, 1994, Anonymous, 2001)

The vein islet number, vein terminal number, stomatal number and stomatal index were determined on fresh leaves using standard procedures.

A. VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER:

The term vein islet in used to denote the minute area of photosynthetic tissue encircled by the ultimate divisions of the conducting strands. The number of vein islets per sq.mm. Area is called vein islet number.

Vein terminal number may be defined as the number of vein terminals present in one sq., mm. area of the photosynthetic tissue.

B. DETERMINATION OF VEIN ISLET NUMBER AND VEIN TERMINATION NUMBER:

Small square portion from the lamina region of the leaf was cleared in chloral hydrate, stained and mounted on a slide. A camera Lucida is set up and by means of a stage micro meter the paper is divided into squares of $1mm^2$using a 16mm objective. The stage micro meter is then replaced by the cleared preparation and the veins are raced in four continuous squares, either in a square 2mm x 2mm (or) rectangle 1mm x 4 mm.

When counting, it is convenient to number each vein-islet on the tracing. Each numbered area must be completely enclosed by veins, and those which are incomplete are excluded from the count if cut by the top and left-hand sides of the square (or) rectangle but included if cut by the other two sides. Ten readings for vein islet and vein termination number were recorded.

C. STOMATAL INDEX:

It is the percentage, which the numbers of stomata from the total number of epidermal cells, each stoma being counted as one cell.

I. **Stomatal index = S/S+E x 100**

Where, S = Number of stomata per unit area

E = Number of epidermal cells in the same unit area

D. DETERMINATION OF STOMATAL INDEX:

The procedure adopted in the determinations of stomatal number was observed under high power (45 X).The epidermal cells and the stomata were counted. From these values the stomatal index was calculated using the above formula.

4.2.6 PHYSICOCHEMICAL PARAMETERS: (Anonymous, 1996, 1998, 2001)

DETERMINATION OF ASH VALUES:

ASH VALUE:

The ash values were determined by using air dried powder of the leaf as per the official method.

TOTAL ASH:

Two grams of the air dried leaf powder was accurately weighed in a silica crucible separately. The powder was scattered into a fine even layer on the bottom of the crucible and incinerated by gradually increasing the temperature not exceeding 450^{o}c, until free from carbon. Then it was cooled and weighed for constant weight. The percentage of ash with reference to the air dried powder was calculated.

WATER SOLUBLE ASH:

The ash obtained from the total ash procedure was boiled with 25ml of water for 5 minutes and the insoluble matter was collected on an ash less

filter paper and washed with hot water. Then it was ignited for 15 minutes at a temperature not exceeding 450^{o}c. The weight of the insoluble matter was subtracted from the weight of the total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash was calculated with reference to the air dried powder.

ACID INSOLUBLE ASH:

The ash obtained from the total ash was boiled for five minutes with 25ml of dilute hydrochloric acid. The insoluble matter was collected in a tarred sintered glass crucible. The residue was washed with hot water, dried and weighed. The percentage of acid insoluble ash with reference to the air dried drug was calculated.

DETERMINATION OF LOSS ON DRYING

For the determination of loss on drying, the method described by Wallis was followed. One gram of dried powdered leaf was accurately weighed in a tarred Petri dish, previously dried under the conditions specified in IP'96. The powder was distributed as evenly as practicable, by gentle sidewise shaking. The dish was dried in an oven at 100 – 105˚C for 1 hour. It was cooled in desiccators and again weighed. The loss on drying was calculated with reference to the amount of the dried powder taken.

EXTRACTIVE VALUES

PETROLEUM ETHER SOLUBLE EXTRACTIVE VALUE

Five gram of the coarsely powder was macerated separately with 100ml of petroleum ether in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it

was filtered rapidly taking precaution against loss of petroleum ether. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the petroleum ether soluble extractive value was calculated with reference to the air dried powder.

ETHANOL SOLUBLE EXTRACTIVE VALUE

Five gram of the coarsely powder was macerated separately with 100ml of ethanol in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of ethanol. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the ethanol soluble extractive value was calculated with reference to the air dried powder.

WATER SOLUBLE EXTRACTIVE VALUE:

Five gram of the coarsely powder was macerated separately with 100ml of chloroform water in a closed flask for 24 hrs. It was frequently shaken for the first 6 hours and allowed to stand for 18 hours. Thereafter it was filtered rapidly taking precaution against loss of chloroform water. 25ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105^{o}C and weighed. The percentage of the chloroform water soluble extractive value was calculated with reference to the air dried powder.

4.3 PHYTOCHEMICAL STUDIES:

[Anonymous, 1998, Chaudhri RD, 1999, Kokate CK, 2005, Agarwal, 2007, Horbone JB, 1973].

4.3.1 PRELIMINARY PHYTOCHEMICAL SCREENING:

TEST FOR ALKALOIDS:

VARIOUS PROCEDURES TO LIBERATE ALKALOIDS:

- Powdered drug was mixed thoroughly with 1ml of 10% ammonia solution and then extracted for 10 minutes with 5 ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was mixed thoroughly with 1ml of 10% sodium carbonate solution and then extracted for 10 minutes with 5ml methanol, under reflux. The filtrate was then concentrated.
- Powdered drug was ground in a mortar for about 1 minute with 2ml of 10% ammonia solution and then thoroughly mixed with 7 gram basic Aluminium oxide. The mixture was then loosely packed into a glass column and 10ml chloroform was added, eluted, dried and methanol was added.
- Powdered drug was shaken for 15 minutes with 15ml of 0.1 N sulphuric acid and then filtered. The filter was washed with 0.1 N sulphuric acid to a volume of 20ml filtrate; 1ml concentrated ammonia was then added. The mixture was then shaken with two portions of 10ml diethyl ether. The ether was dried over anhydrous sodium
- Sulphate, filtered and evaporated to dryness and the resulting residue was dissolved in methanol.
- Powdered drug was mixed with one gram of calcium hydroxide and 5ml of water, made into a smooth paste and set aside for 5 minutes. It was then evaporated to dryness in a porcelain dish on a water

bath. 20ml of 90% alcohol was added, mixed well and then refluxed for half an hour on a water bath. It was then filtered and the alcohol was evaporated. To that dilute sulphuric acid was added.

- The above made extracts were tested with various alkaloid reagents as follows.

1. MAYER'S REAGENT

2. DRAGENDORFF'S REAGENT

3. HAGER'S REAGENT

4. WAGNER'S REAGENT

TEST FOR CARBOHYDRATES:

MOLISCH'S TEST:

- The aqueous extract of the powdered material was treated with alcoholic solution of α - naphthol in the presence of sulphuric acid**.**

FEHLING'S TEST:

- The aqueous extract of the powdered material was treated with Fehling's I and II solution and heated on a boiling water bath.

BENEDICT'S TEST:

- The aqueous extract of the powdered drug was treated with Benedict's reagent and heated over a water bath.

TEST FOR GLYCOSIDES:

GENERAL TEST:

TEST A:

200 mg of the powdered drug was extracted with 5ml of dilute sulphuric acid by warming on a water bath, filtered and neutralized with 5% sodium hydroxide solution. Then 0.1ml of Fehling's solution I and II were added, until it becomes alkaline and heated on a water bath for 2 minutes.

TEST B:

200 mg of the powdered drug was extracted with 5ml of water instead of sulphuric acid. Boiled and equal amount of water was added instead of sodium hydroxide solution. Then 0.1ml of Fehling's solution I and II were added, until it becomes alkaline and heated on a water bath for 2 minutes.

ANTHRAQUINONES:

BORNTRAGER'S TEST:

The powdered leaf was boiled with dilute sulphuric acid, filtered and to the filtrate benzene was added and shaken well. The inorganic layer was separated and ammonia solution was added slowly.

MODIFIED BORNTRAGER'S TEST:

About 0.1gram of the powdered leaf was boiled for two minutes with dilute hydrochloric acid and few drops of ferric chloride solution was added, filtered while hot and cooled. The filtrate was then extracted with benzene and the benzene layer was separated. Equal volume of dilute ammonia solution was added to the benzene extract and shaken well.

TEST FOR CYANOGENETIC GLYCOSIDES:

Small quantity of the powdered leaf was placed in a stoppered conical flask with just sufficient water to cover it. A sodium picrate paper strip was inserted through the stopper so that it was suspended in the flask and it was set aside for 2 hours in a warm place.

TEST FOR CARDIAC GLYCOSIDES:

KELLER KILLIANI'S TEST:

About 1gram of the powdered leaf was boiled with 10ml of 70% alcohol for two minutes, cooled and filtered. To the filtrate 10ml of water and 5 drops of solution of lead sub acetate were added and filtered. The filtrate was then extracted with chloroform and the chloroform layer was separated and evaporated to dryness. The residue was dissolved in 3ml of glacial acetic acid containing a trace of ferric chloride. To this 3ml of concentrated sulphuric acid was added along the sides of the test tube carefully.

RAYMOND TEST:

To the alcoholic extract of the leaf, hot methanolic alkali was added.

LEGAL'S TEST:

To the alcoholic extract of the powdered drug, pyridine and alkaline sodium nitro pruside solution were added.

COUMARIN GLYCOSIDES:

A small amount of powdered leaf was placed in test tube and covered with a filter paper moistened with dilute sodium hydroxide solution. The

covered test tube was placed on water bath for several minutes. Then the paper was removed and exposed to UV light.

TEST FOR PHYTOSTEROLS:

The powdered leaf was first extracted with petroleum ether and evaporated. The residue obtained was dissolved in chloroform and tested for sterols.

SALKOWSKI TEST:

Few drops of concentrated sulphuric acid were added to the above solution, shaken well and set aside.

LIBERMANN – BURCHARD'S TEST:

To the chloroform solution few drops of acetic anhydride was added and mixed well. 1ml of concentrated sulphuric acid was added through the sides of the test tube and set aside for a while.

TEST FOR SAPONINS:

About 0.5gram of the powdered leaf was boiled gently for 2 minute with 20 ml of water and filtered while hot and allowed to cool. 5ml of the filtrate was then diluted with water and shaken vigorously**.**

DETERMINATION OF FOAMING INDEX:

One gram of the coarsely powdered leaf was weighed and transferred to 500 ml conical flask containing 100 ml of boiling water. The flask was maintained at moderate boiling, at 80-90^{o}C for about 30 minutes. Then it was cooled and filtered into a volumetric flask and sufficient water was added through the filter to make up the volume to 100 ml (V_1).

Ten Stoppard test tubes were cleaned (height 16 cm, diameter 1.6 cm) and marked from 1 to 10. Measured and transferred the successive portions of 1, 2, 3ml up to 10ml and adjusted the volume of the liquid in each tube with water to 10ml. Then the tubes were Stoppard and shaken lengthwise for 15 seconds, uniformly and allowed to stand for 15 minutes and measured the length of the foam in every tube.

TEST FOR TANNINS:

To the aqueous extract of the powdered leaf, few drops of ferric chloride solution were added.

GOLD BEATER'S SKIN TEST:

2% hydrochloric acid was added to a small piece of gold beater skin and rinsed with distilled water and placed in the solution to be tested for five minutes. Then washed with distilled water and transferred to a 1% ferrous sulphate solution.

TEST FOR PROTEINS AND FREE AMINOACIDS:

MILLON'S TEST:

The acidulous alcoholic extract of the powdered leaf was heated with Millon's reagent.

BIURET TEST:

To the alcoholic extract of the powdered leaf 1ml of dilute sodium hydroxide was added. Followed by this one drop of very dilute copper sulphate solution was added.

NINHYDRIN TEST:

To the extract of the powdered drug, Ninhydrin solution was added, and boiled.

TEST FOR MUCILAGE:

To the aqueous extract of the powdered leaf, Ruthenium red solution was added.

TEST FOR FLAVONOIDS

SHINODA TEST:

A little amount of the powdered leaf was heated with alcohol and filtered. To the alcoholic solution a few magnesium turnings and few drops of concentrated hydrochloric acid were added, and boiled for 5 minutes.

ALKALINE REAGENT TEST:

To the alcoholic extract of the powdered leaf, few drop of sodium hydroxide solution was added.

ZINC HYDROCHLORIDE TEST:

To the alcoholic extract, mixture of zinc dust and concentrated Hydrochloric acid was added.

TEST FOR TERPENOIDS:

The powdered leaf was shaken with petroleum ether and filtered. The filtrate was evaporated and the residue was dissolved in small amount of chloroform. To the chloroform solution tin and Thionyl chloride were added.

TEST FOR VOLATILE OIL:

About 100gram of fresh leaves, were taken in a volatile oil Clevenger apparatus and subjected to hydro distillation for four hours.

TEST FOR FIXED OIL:

A small amount of the powdered leaf was pressed in between in the filter paper and the paper was heated in an oven at 105^{o}C for 10 minutes.

4.3.2. FLUORESCENCE ANALYSIS:

Powdered leaf material Of*P. nigrum* was subjected to analysis under UV light after treatment with various chemical and organic reagents like Ethanol, Ethyl acetate, Chloroform, Water, 50% sulphuric acid, 10% sodium hydroxide, 50% nitric acid and dried leaf powder. (Horbone JB, 1973).

4.3.3. ESTIMATION OF FLAVONOID CONTENT:

[Chang CC *et al*., 2002, Mabry TJ*et al*., 1970 and Siddiquie MA *et al.,* 2010]. The flavonoid content of plant extract was estimated by aluminium chloride method. In this method, aluminium chloride complexes with flavonoids of C3-C5 hydroxyl group and to produce intense colour in acidic medium. The intensity of the colour is proportional to the amount of flavonoids and can be estimated as quercetin equivalent at wavelength of 415nm.

MATERIALS REQUIRED:

- Ethanolic extract of leaves of *S.aromaticum*
- 10%w/v aluminium chloride
- 1M Potassium acetate
- 95%v/v ethanol

PROCEDURE:

0.5ml of the extract (1mg/ml) was transferred to a test tube. To this solution, 0.1ml of aluminium chloride, 0.1ml of potassium acetate, 1.5ml ethanol were added and made up to 5ml with distilled water. The mixture was allowed to stand for 30 min with intermittent shaking. The absorbance was measured at 415nm. The calibration curve was generated using quercetin as a standard at different concentrations (5-50µg/ml). The reaction mixture without aluminium chloride was used as a blank. The flavonoids content was expressed as mg of quercetin equivalent per gram of extract.

4.3.4. ESTIMATION OF TOTAL PHENOLIC CONTENT

(Singleto VL *et al*., 1979, Gouthamchandra K *et al.,* 2010)

PRINCIPLE:

The total phenolic content of the extract was determined by Folin&Cio-calteu's phenol reagent. This reagent consists of phosphotungstate and phosphomolybdate mixture which is reduced to mixture of blue molybdenum and tungsten oxides while phenolic content of the extract was oxidized. The intensity of colour is proportional to the amount of phenolic content of the extract and which was measured at 765nm. The total phenolic content in the extract was expressed as milligrams of gallic acid equivalent (GAE) per gm of extract.

MATERIALS:

- Ethanolic extract of leaves of*S.aromaticum*
- 10%w/v sodium carbonate solution
- Gallic acid
- Folin&Cio-21021calcateu's phenol reagent

PROCEDURE:

0.5ml and 1ml of extract was transferred into separate test tube. To this solution, FCR 0.5ml and 1ml of sodium carbonate were added and final volume made up to 10ml with distilled water. The mixture was allowed to stand for 1hr with intermittent shaking. The absorbance was measured at 765nm. A calibration curve was generated using Gallic acid as a standard at different concentrations (2, 4, 6, 8, 10µg/ml). The reaction mixture without sample was used as a blank. The total phenolic content was expressed as milligrams of Gallic acid equivalent (GAE) per g of extract.

4.3.5 DETERMINATION OF TRACE ELEMENTS IN THE LEAF OF *Syzygium aromaticum* BY ENERGY DISPERSIVE X-RAY SPECTROMETER (EDS):

The SEM allows the observation of materials in macro and submicron ranges. SEM is capable of generating 3-D images for analysis of topographic features. When SEM is used along with EDS the analyst can perform an elemental analysis on specimens of microscopic sections or contaminants that may be present.

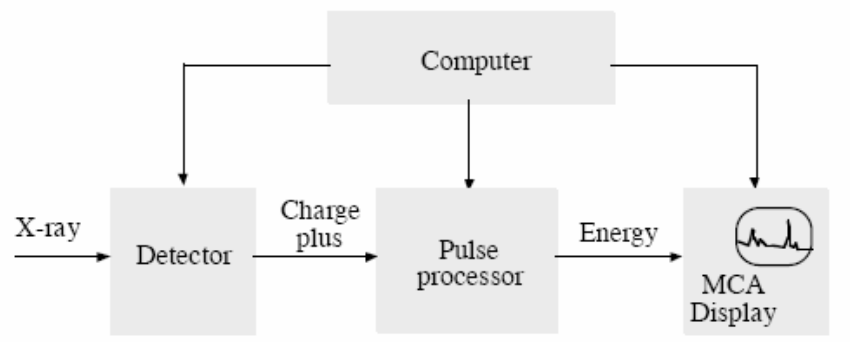
EDS ANALYTICAL CAPABILITIES:

Backscattered electron images in the SEM display compositional contrast that results from different atomic number elements and their distribution. EDS is used to find particular elements are and their Atomic %. The Y-axis shows the counts (number of X-rays received and processed by the detector) and the X-axis shows the energy level of those counts [Bob Hofner].

By Viewing 3-D images of specimens solves some of the problem in an analysis and it is also necessary to detect different elements associated with the specimen. This is accomplished by using the “built-in” spectrometer called an Energy Dispersive X-ray Spectrometer.

EDS SYSTEM COMPRISES OF 3 BASIC COMPONENTS:

- An X-ray Detector - detects and converts X-ray into electronic signals.
- A Pulse Processor - measures the electronic signals to find out energy of each X-ray detected; and
- A Multiple Channel Analyser - interprets and displays analytical data.



EDS is an analytical technique in which the specimen emits X-rays due to the bombardment of electron beam on it which is used to identify the elemental composition of the specimen due to the ejection of electrons from the atoms on the specimen surface. To explain further, when the sample is bombarded by the electron beam of the SEM, electrons are ejected from the atoms on the specimen’s surface. A resulting electron vacancy is filled by an electron from a higher shell, and an X-ray is emitted to balance the energy difference between the two electrons. The EDS X-ray detector measures the number of emitted X-rays emitted versus their energy.The energy of the X-ray is characteristic of the element from which the X-ray was emitted. A spectrum

of the energy versus relative counts of the detected x-rays is obtained and evaluated for the determinations of the elements.

4.3.6 IDENTIFICATION OF COMPOUNDS PRESENT IN THE VOLATILE OIL OF LEAVES BY GC-MS ANALYSIS.

GAS CHROMATOGRAPHY

GC now ranks as the most important technique in analytical chemistry because of its several advances in its instrumentation. GC requires the vapourization of sample at the injection point which is carried by carrier gas (mobile phase) at a suitable temperature and pressure. The carrier gas which passes through the injection point is heated to the temperature of stationary phase (column) or heated injection block or if flash heater is used to about 50ºC above that of the column. The sample must be stable when vaporized and also its passage through the packed column, in order to avoid the production of complex chromatogram (carrier gas elutes the product from the column) and also when vaporized. The instantaneous vapourisation of sample and the detector produces an electrical output proportional to the amount of compound emerging from the column.

MASS SPECTROMETRY

Wien, in 1898, produced the first crude mass spectra when he demonstrated that positive ions could be deflected according to their masses in electric or magnetic fields. This observation was developed by Thompson (1910) who used combined electrostatic and a magnetic field to observe the mass spectrum of mixture of rare gases.

In single focusing mass spectrometer the sample is introduce into the instrument in such a way that its vapour is bombarded by electrons having an energy of about 70eV. Positive ions formed in ion source are accelerated between two plates by potential difference of a few thousand volts (V). The ions pass through the source slit and are deflected by magnetic field (H) according to their mass/charge ratios. They then pass through the exit or collector slit and impinge upon the collector; the signal received is amplified and recorded. The height or intensity of the resulting peak is proportional to the ion abundance.

COMBINATION OF GC WITH MASS SPECTROMETRY

The identification of fractions in gas chromatography is essentially comparative, in that the characteristic of the unknown are compared with those of known library compound. By correct choice of column, the fraction consists of single substance only, so that, if each is examined by other methods for identification, a powerful analytical tools becomes available. This may be done in several ways and GC is now used in conjuction with IR spectra and mass spectra.

Gas liquid chromatography is a very effective method for separating a complex mixture into its individual components. The high sensitivity of mass spectrometry provides the necessary information for either identification of compounds by comparison with available spectra or structural elucidation of small quantities of compounds. Gases and volatile liquids are admitted to the source through a small leak from the gas reservoir.Hence GC-MS is the introduction of GC effluents without most of carrier gas into a mass spectrometer has its increasing utility in structural organic chemistry,

pharmaceutical analysis and biochemistry. Here the fractions which elutesfrom GC column is condensed into a capillary or onto a small metal surface and this fractions are introduced into the MS source. For multicomponent mixture many operations are involved and losses may occur during the collection of fractions; however, the mass spectrometer may be operated at high resolution and GC carrier gas is admitted to the instrument.

GC/MS combination produce a wealth of data rapidly. To process and interpret all of this data manually would be excessively time consuming.

Instrument Name	:	JEOL GC MATE 11
Front inet temp	**:**	220^{o}C
Column	:	HP 5 Ms
Carrier gas	:	High Pure Helium Gas
Flow Rate	:	1 ml/ min
Oven Tem	:	50 to 250 @ 10 deg /min
Ion chamber temp	:	250 ^{o} C
GC interface Tem	:	250^{o} C
Mass analyser	:	Quadrupole with double focusing massanalyser
Detector	:	Photon Multiplier Tube
Scan	:	50 to 600 amu 70 ev

4.3.7 Preparation of nanospheres of volatile oil of the leaves of *S.aromaticum* (SALVONS):

VO of the *S.aromaticum* leaves is encapsulated in to a 1:1(w/w) polymer blend of MC and EC at a VO to EC/MC polymer weight ratio of 1:1 by

displacing the ethanol solvent with water, as reported (Sansukcharearnpon et al., 2010). To this end, the two polymers (125mg EC and 125 mg MC) were dissolved in 25 ml of 75% (v/v) aqueous ethanol at 70^{o} C, allowed to cool to room temperature and then (250mg) added and dispersed. After mixing water was slowly dropped (0.75ml/min) in to the mixture to a final volume of 100ml. To determine the encapsulation efficience (EE) and loading capacity the suspension was filetered , centrifuged through a 100,000 Da MW cut off membrane at 9392X g for 10 min and the obtained clear supernatant was quantified for eugenol contentby UV spectrophotometry, measuring the absorbance at a wavelength of 275nm with refernece to a freshly prepared calibration curve. The EE and VO loading level were then determined as follows:

- % EE=Wt of VO in spheres /Wt of VO used X 100
- % Loading level =Wt of VO in spheres /Wt of VO in spheres+ Wt of polymerX 100

4.3.8 Characterisation of nanospheres:

The aqueous suspension of VO loaded particles was subjected to analysis by SEM.

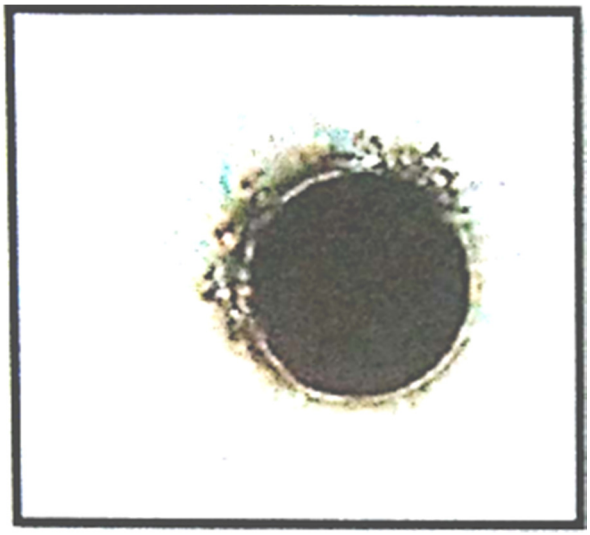
4.4 PHARMACOLOGICAL STUDIES:

4.4.1 ACUTE TOXICOLOGICAL STUDY USING BRINE SHRIMP LETHALITY ASSAY (BSLA):

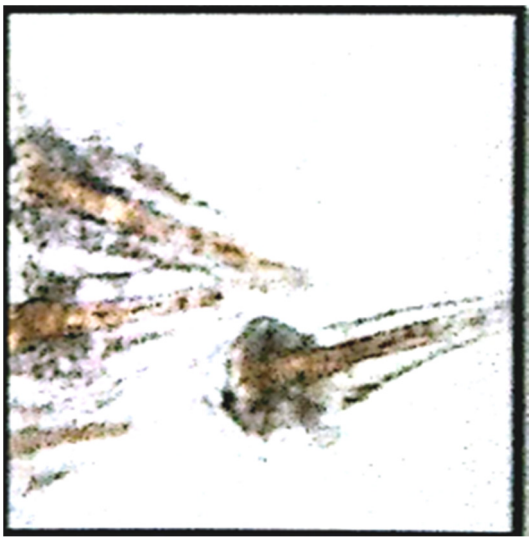
(Michael AS *et al.,* 1956, Vanhaecke P *et al.,* 1981, Sleet RB and Brendel K., 1983)

FIGURE 8

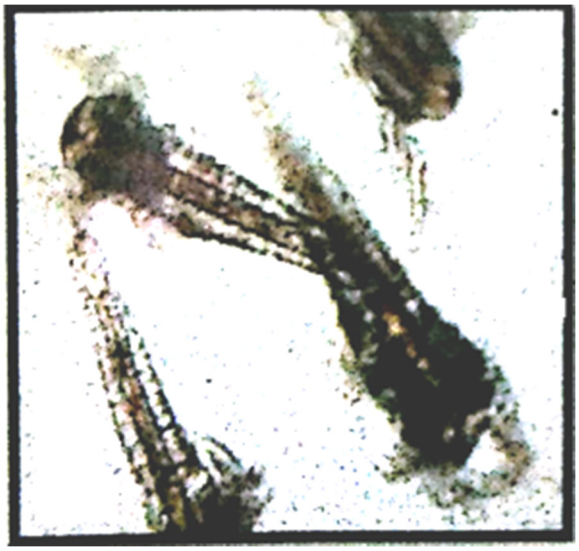
LIFE CYCLE OF ARTEMIA NAUPLII



CYST



1ST DAY LARVAE



2ND DAY

3RD DAY

The importance of medicinal plants and traditional health systems solving the health care problems of the world is gaining increasing attention. Most of the developing countries have adopted traditional medical practice as an integral part of their culture.

In order to study the toxicity of these medicinal plants we performed Brine Shrimp Lethality Bioassay which based on the ability to kill laboratory cultured brine shrimp (*Artemia nauplii*). The brine shrimp assay is a useful tool for preliminary assessment of toxicity and it has been used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticides and cytotoxicity testing of dental materials.

The method is attractive because it is very simple, inexpensive and low toxin amount are sufficient to perform the test in the micro well scale.

PRODUCTION OF *Artemia* nauplii:

Artemia a non-selective filter feeder of organic detritus, micro algae and bacteria. Artemia are naturally found in saltpans, hyper saline lakes and coastal lagoons as well as in the manmade saltpans. When the cysts are inoculated in seawater for 24hrs the free-swimming nauplii are hatched out.

CYTOTOXICITY BIOASSAY:

Ten nauplli were drawn through a glass capillary and placed in each vial containing 4.5ml of brine solution. In each experiment, 0.5ml of the extract was added to 4.5 ml of brine solution and maintained at room temperature for 24hrs under the light and surviving larvae were counted. Experiment was conducted along with control (vehicle treated), different concentrations of the aqueous extract (100-7500 ppm) in a set of three tubes per dose.

LETHALITY CONCENTRATION DETERMINATION:

The percentage lethality was determined by comparing the mean surviving larval of the test and control tubes. LC_{50} value was obtained from the best – fit line, plotted concentration verses percentage lethality. Podophyllotoxin was used as a positive control in the bio assay.

ABBOT'S FORMULA:

$$\text{CORRECTED MORTALITY}(\%) = \frac{\text{TEST MORTALITY}(\%) - \text{CONTROLMORTALITY}(\%)}{100 - \text{CONTROL MORTALITY}(\%)} \times 100$$

4.4.2. EFFECT OF VOSAL ON MUTAGENESIS OF *Drosophila melanogaster* (Frei.H&Wurgler.E.F 1998).

Due to its lack of information about its mutagenic effect,So it is important to evaluate the effect on mutagenic alterations. Over the last few years, mutagenic assays have been developed that are able to detect several genetic endpoints. In genetic toxicology, it is important to know whether chemical should regarded as hazardous or whether they can be considered as sufficiently below a defined minimal effect level.

The eye and wing spot assays have received increasing interest in the recent past. Because they are sensitive in vivo assays which are simple to carry out. They are also much less laborious, cheaper, and at the same time more informative.

In addition it is well established that *Drosophila* possess versatile system and has proved to be an assay for mutagenic screening which is easy to perform and inexpensive for the detection of promutagens.

If a mutagenic alteration takes place in one of the cells, the descendent cells will form a clone of mutant cells that can be detected as a spot in the adult tissue.

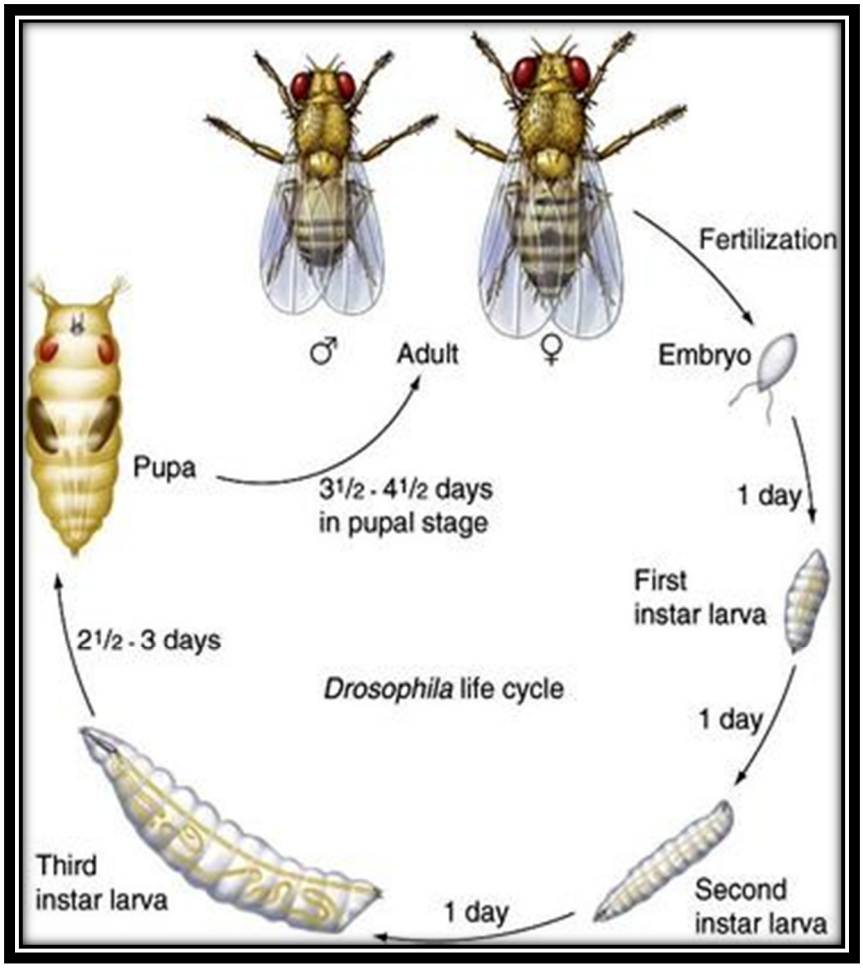
The test can detect the mutagenic chemical compound that produced a loss of heterozygosis by several chromosome breakage mechanisms such as mitotic recombination, deletion, point mutation, chromosomal loss and aberration of *Drosophila melanogaster*: (AsbhurnerM, Wright TRF 2005, Golic KG, Hawley RS 2005)

Drosophila is a genus of small flies, belonging to the family Drosophilidae, whose members are often called “fruit flies” or more appropriately (though less frequently) pomace flies, vinegar flies, or wine flies, a reference to the characteristic of many species to linger around overripe or rotting fruit. These feed primarily on unripe or ripe fruit, with many species being regarded as destructive agricultural pests, especially the Mediterranean fruit fly. One species of *Drosohila* in particular, *D.melanogaster*, has been heavily used in research in genetics and is a common model organism in developmental biology. Indeed, the terms “fruit fly” and “*Drosophila*” are often used synonymously with *D.melanogaster*in modern biological literature.

PHYSICAL APPEARANCE OF *D.melanogaster*:

Fruit flies have brick red eyes, are yellow-brown in colour, and have transverse black rings across their abdomen. They exhibit sexual dimorphism; Females are about2.5millimeters (0.1 inches) long; males are slightly smaller and the back of their bodies is darker. Males are easily distinguished from females based on colour differences, with a distinct black patch at the abdomen, less noticeable in recently emerged flies, and the sex combs (a row

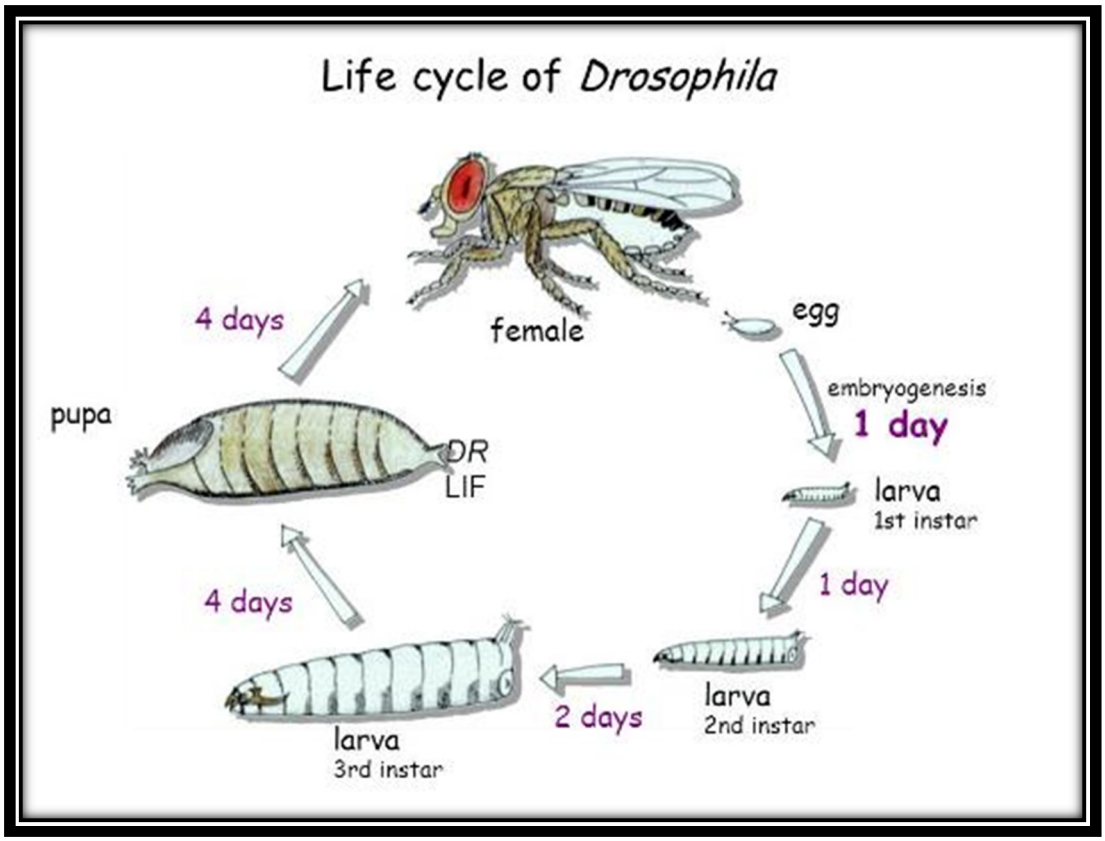
LIFE CYCLE OF *Drosophila melanogaster*



FORMALIN TREATED *Drosophila melanogaster*

DIAGRAMMATIC REPRESENTATION OF LIFE CYCLE OF

Drosophila melanogaster



of dark bristles on the tarsus of the first leg). Furthermore, males have a clusterof spiky hairs (claspers) surrounding the reproducing parts used to attach to the female during mating.

LIFE CYCLE AND REPRODUCTION OF *D.melanogaster*:

The developmental period for *D. melanogaster* varies with temperature, as with many ectothermic species. The shortest development time (egg to adult), 7 days is achieved at 28^{o}C. Development time increase at higher temperatures 30^{o}c, 11 days due to heat stress. Under ideal conditions, the development time at 25^{o}C (77^{o}F) is 8.5 days. Under crowed conditions, development time increases, while the emerging flies are smaller. Female lay some 400 eggs (embryos), about five at a time, in to rotting fruit. The eggs, which are about 0.5 millimetre long, hatch after 12-15 h (at 25^{o}C (77^{o}F)). The resulting larvae grow for about 4 days (at 25^{o}C) while molting twice (into 2nd- and 3rd-instar larvae), at about 24 and 48 h after hatching. During this time, they feed on the microorganisms that decompose the fruit, as well as on the sugar of the fruit itself. Then the larvae encapsulate in the puparium and undergo a four-day-long metamorphosis (at 25^{o}C), after which the adults eclose (emerge).

MODEL ORGANISM: (Adams MD*etal.,*2001)

Drosophila melanogaster is one of the most studies organisms in biological research, particularly in genetics and developmental biology.

THERE ARE SEVERAL REASONS:

The care and culture requires little equipment and use little space even when using large cultures, and the overall cost is low.

- It is small and easy to grow in the laboratory and their morphology is easy to identify once they are anesthetized (usually with ether, carbon dioxide).
- It has a short generation time (about 10 days at room temperature) so several generations can be studied within a few weeks.
- It has high fecundity (females can lay more than 800 eggs in a life time, i.e. one egg every 30 minutes with sufficient food).
- Males and females are readily distinguished and virgin flies are easily isolated, facilitating genetic crossing.
- It has only four pairs of chromosomes: three autosomes, and one sex chromosome.
- Genetic transformation techniques have been available since 1987.
- Its complete genome was sequenced and first published in 2000.

SIMILARITY TO HUMANS:(Bloomington *Drosophila* Stock center at Indiana University)

About 75% of known human disease genes have a recognizable match in the genetic code of fruit flies and 50% of fly protein sequences have mammalian analogues. An online database called homophilia is available to search for human disease gene homologues in flies and vice versa. *Drosophila* is being used as genetic model for several human diseases including the neurodegenerative disorders Parkinson's, Huntington's, spinocerebellar ataxia and Alzheimer's disease. The fly is also being used to

study mechanisms underlying aging and oxidative stress, immunity, diabetes and cancer as well as drug abuse.

MATERIALS AND METHODS:

- Stereomicroscope
- Micropipettes
- Diethyl ether
- Stage micrometer
- Eyepiece micrometer
- Formaldehyde (0.5%V/V)
- VOSAL the concentration of 1, 2 and 4µl/ml conc
- Plastic containers
- 70%V/V Ethanol
- Standard fly food
- Petridishes.

EXPERIMENTATION:

1. IInd in star larvae from F1 generation of normal flies were collected.
2. Larvae were washed with solution of 20% W/V sucrose and seeded in glass petridishes (20 larve/vial) containig 2 gm of fly food with 1ml of total alkaloid extract of various concentration (10, 20 µg/ml) along with negative control of solvent alone (distilled water) and standard chemical mutagen(0.5% V/V formalin).
3. Larvae were fed on the above medium for six hours and transferred to fresh medium for the rest of their development.

4. After eclosion, adult flies were collected and stored in70%V/V ethanol for the evaluation for the evaluation of the mutagenic effect.
5. Morphological changes including eye colour, spots in the wings, wing hairs, changes in the length and width of the wing, wing shape, abdomen length and total body length were observed under stereomicroscope.
6. All the experiments were carried out at room temperature (±12h day and night).

REPRESENTATIVE *Drosophila* MODELS OF ALZHEIMER'S DISEASE:

Drosophila has emerged as an excellent model system for neurodegenerative diseases including Alzheimer'sdisease (AD) and Parkinson's disease (PD) because of genetic homology, ease of genetic manipulation, and well-conserved disease-associated genes. *Drosophila* geneticists have successfully used these models to identify many diseases associated genes, which sheds light on our understanding of the pathology of these diseases.

Alzheimer disease (AD) is the most common neurodegenerative disease, which causes a deficiency in memory and cognitive functions. The chief event in AD pathogenesis is the accumulation of amyloid β-peptide 42 (Aβ42), a form of β-amyloid precursor protein (APP) proteolytically processed byγ-secretase. Aggregates of abnormally phosphorylated, a microtubule-binding protein, tau are also shown to be closely associated with neuronal loss in AD. The *Drosophila* genome contains genes that encode orthologs of APP, tau, and four major protein components of γ-secretase (presenilin, nicastrin, APH-1, and PEN-2). Transgenic flies expressing human *Aβ42* or *tau*

ectopically developed late onset neuronal degeneration and had a shortened lifespan. *Drosophila* AD models present various easilyvisible and quantifiable phenotypes such as eye degeneration, locomotor defects, shortened lifespan, developmental defects, learningand memory defects increased oxidative stress sensitivity, which make it suitable for *in vivo* genetic screening.

The experimental methods foranalysing these phenotypes are described as: Based on genetic screening using *Drosophila* models of AD, several biochemical processes such as secretion, regulation of chromatin structure, and cholesterol homeostasis have been found to be involved in mediating the toxic effects of Aβ42.

In *tau*-expressing models, kinases and phosphatases comprised the major classes of modifiers ofthetauopathy, and cytoskeleton proteins and molecular chaperones have been identified as modulators of mutant tau-induced neurodegeneration. More recently, DNA damage-activated checkpoint kinase 2, histone deacetylase6, and epidermal growth factor receptor (EGFR) have been reported to be implicated in AD pathologies in *Drosophila* AD models. Moreover, several *in vivo* reporter systems for measuringAPPγ-secretase activity were developed in *Drosophila*. Among them, the transgenic system consisting of the human APP and the yeast GAL4 fusion protein under the expression of the eye-specific *glass multimer reporter* (*GMR*) promoter has been applied as a powerful genetic screening tool for isolating γ-secretase activity-regulating molecules. In this, the presence of γ-secretase activity, the intracellular domains of APP and GAL4 translocate to the nucleus and induce *GRIM* expression, which results in cell death in the eye. Therefore, genetic or pharmacological modulators of γ-

secretase activity can be screened by simply observing the eye degeneration phenotype. Several genetic modulators of γ-secretase activity were also identified using this reporter system.

REPRESENTATIVE NEUROLOGICAL PHENOTYPES OF *Drosophila* MODELS OF AD AND PD:

Neurodegenerative disease models of *drosophila* show a variety of phenotypes, which are very similar to the symptoms of human patients and closely linked with the neuropathology of the diseases. These phenotypes include a wide range of biological processes, from cellular phenotypes to behaviours. These prominent and easily observable phenotypes make *Drosophila* a valuablemodel for drug screening and discovery of novel disease associatedgenes.

Accumulation of *Aβ42* andα-syn*.* One of the major characteristics of AD is the accumulation of amyloid protein in the cerebral cortex. Mutation in *APP*, *Presenilin 1*, and *Presenilin 2* genes or other factors increases Aβ42 production and accumulation. Consequently, increased Aβ42 oligomerization and deposition injure the neurons, causing neuronal dysfunction and cell death.

These AD-like phenotypes can also be observed in *Drosophila* AD models. Over expression of *Aβ42* in the nervous system induces neuronal loss accompanied by the accumulation of Aβ42 in the adult brain. Similarly, PD as a neurodegenerative disease is characterized by the loss of nigrostriatal DA neurons and the accumulation of Lewy bodies in neurons. Over expression of α-*syn*gene in the nervous system of the fly model results

in the death of DA neurons and the formation of Lewy body-like filamentous intraneuronal inclusions.

Eye Degeneration. Although the central nervous system is the main target of neurodegenerative diseases such as AD, PD, and Huntington's disease (HD), functional defects in these diseases are not restricted to the brain. For example, extensive ganglion cell loss was observed in the central retina of AD patients, and visual dysfunction caused by retinal degeneration has been found in multiple transgenic AD mouse lines. Thus, tissues other than that of the brain can be used to identify the function of genes related to neurodegenerative diseases. Eyes are prominent organs in the body of *Drosophila*. Therefore, an ocular phenotype is easily distinguishable and facilitates simple, easy, and efficient genetic or pharmacological screening. Moreover, the developing *Drosophila* eye contains the photoreceptor neurons. *Drosophila* has two compound eyes, each consisting of about 800 ommatidia and bristles. These ommatidia are arranged very regularly. Using the *UAS-GAL4* system, the expression of a human disease-related transgene in the fly eye creates a fly model for neurodegenerative disease as well as helps to discover the function of the gene. For example, overexpression of the *Aβ42* and *tau* genes involved in AD or the α*-syn*gene involved in PD induces apoptotic eye degeneration, reduced eye size, and deformed ommatidia.

***Drosophila* strains:**

The strains used in the study Aβ42 expressing *Drosophila melanogaster.*

4.4.3 *In vivo* EFFECT ON THE TRANSGENIG *Drosophila melanogaster* MUTANT WITH Aβ42 INDUCED NEURODEGENERATION OF VOSAL

4.4.3.1 EFFECT OF VOSAL ON LONGEVITY OF Aβ42 EXPRESSING *Drosophila* (Fai C Ng *et al.,* 2013)

n= 30

Normal control- Not expressing Aβ maintained at the normal diet Aβ expressing control – Maintained on the normal diet

Positive control – Diet containing 10 µmol donepezil/gm of *Drosophila* food medium

Treated groups- Diet containing 1, 2 and 4mg leave oil /gm of *Drosophila* food medium

Dead drosophila are counted on day 1 and 5 in a seven days cycle. The feeding lasted for 65 days

4.4.3.2 EFFECT OF VOSAL ON LOCOMOTOR FUNCTION BY CLIMBING ASSAY OF Aβ42 EXPRESSING *Drosophila*(Fai Ng C et al., 2013).

Locomotor function of *drosophila* was measured according to the climbing assay. 30 *drosophila* are placed at the bottom of 15ml stout wall test tube and were given 10sec to climb up the tube. At the end of the trail the number of *drosophila* that climbed up to a vertical distance of 8cm or above was recorded. They were tested on day1 and 5 in a seven day cycle in triplicate.

4.4.3.3 PSEUDOPUPIL TECHNIQUE TO VISUALIZE THE RETINO RHABDOMERES IN ADULT EYES:

The control, positive and Aβ42 were treated as above. *Drosophila* heads were examine under light microscope. The compound eye of 5days old drosophila was viewed under microscope. Each photoreceptor projected, the rhapdomere, in to the center of the ommatidium which are appeared as bright spots and rhabdomeres in each ommatidium were counted (in the control seven rhabdomere could be observed in an each ommatidium) 100 ommatidia were observed from 5-10 eyes and the average rhabdomeres count per ommatidium was calculated. Three trails were contacted for each group

4.4.3.4. EVALUVATION OF SURFACE ORGANIZATION OF OMMATIDIA IN ADULT EYES BY NAIL – POLISH IMPRINTS AND SEM: (Arya R and Lakhotia S C 2006)

Recently, fly models for different human neurological and other genetic disorders was also been developed and widely used for identifying interacting genes and for developing therapeutic strategies . The compound eyes of flies have been particularly useful in this situation because

- The genetic pathways that determine eye development are understood better,
- The adult eyes contain both neuronal and non-neuronal cell types,
- They are dispensable for survival of the flies, and
- The phenotypic consequences of any perturbations in eye development are distinct and quantifiable.

The adult *Drosophila* eye contains about 800 highly ordered matrix ommatidia arrangement with arrays of sensory bristles projecting out from the surface of each ommatidial unit.

To exploit these features of the *Drosophila* eyes researchers use scanning electron microscopy (SEM) to examine any disruption in the ordered arrays of the ommatidia in adult eyes. A novel, simple and inexpensive method that provides high quality images, comparable to those obtained by SEM, of the external surface of eyes of adult flies, but which does not require expensive facility and can be used in any laboratory with a good light microscope was described

In this method, a transparent nail polish is used to create an exact replica of the external surface of the eye, which is subsequently examined using a light microscope. An imprint of adult eye is obtained by a small drop of transparent nail polish placed on the surface of a clean glass slide. The fly to be examined is anaesthetized, placed on a dry area of the slide and decapitated with a sharp blade or needle. The decapitated head is held with forceps or needles and is briefly dipped in the still fluid drop of nail polish. The head is then placed in a clean and dry area of the same slide and the nail polish layer on the eyes is allowed to dry at room temperature (preferably 24°C) for 5– 10 min. The dried layer of nail polish can be easily peeled-off from the eye with the help of fine dissecting needles. The separated peel, being an exact replica of the eye surface, assumes a goblet-shaped appearance. This peel is carefully placed on another clean glass slide with the imprint side facing upright. This nail polish imprint can be directly examined and photographed under a stereo binocular microscope to provide a low

magnification image of the eye surface. For higher magnification and for better image of the eye surface, the peel is carefully flattened by gently placing a cover slip over it and carefully applying a slight pressure. The eye imprint is then examined under a microscope using 10X, or 45X differential interference contrast (DIC) objective. These can be seen with bright field optics as well, but the DIC optics provide a better image. Details of eye surface are generally comparable with those obtained by SEM. The nail polish imprints also easily reveal the characteristic defects due to different mutants affecting eye development.

We performed both nail polish imprint technique and SEM to analyse the external surface of the eye of *Drosophila.*

RESULTS

FIG – 1

DIAGRAM SHOWING THE AERIAL PARTS OF *S.aromaticum*

PLATE 1

HABIT AND HABITAT OF *S. aromaticum*

RESULTS

5.1 PHARMACOGNOSY:

5.1.1 MORPHOLOGICAL FEATURES OF *Syzygium aromaticum* (PLATE 1, FIG 1)

Clove tree is a small ,handsome evergreen tree.,reaching 12-15m in height.

LEAVES : (PLATE 2,3)

Shape	:	Leaves are simple, opposite, coriaceous, exstipulate, glabrous and aromatic
Size	:	7.5-12.3 x2.5-3.75 cm
Color	:	Green
Margin	:	Entire
Base	:	Cuneate
Apex	:	Shortly or broadly blunty acuminate
Petiole	:	2-3 cm long

STEM

The trunk up to 30 cm in diameter is composed of very hard wood.If often forks near the base into two or three main erect branches.

FLOWERS: (PLATE 4)

The inflorescence is a terminal, corymbose,trichotomous panicle, shortly pedunculate and branched from the base. The flower is hermaphrodite with a fleshy hypanthium which is surmounted by the sepals.The four calyx

Plate 2

BRANCH OF *S.aromaticum* SHOWING LEAF ARRANGEMENT

PLATE 3

DORSAL AND VENTRAL VIEW OF *S. aromaticum* LEAF

PLATE 4

FLOWER AND FLOWER BUD OF *S. aromaticum*

PLATE 5

FRUIT OF *S. aromaticum*

PLATE 6

SEED OF S.aromaticum

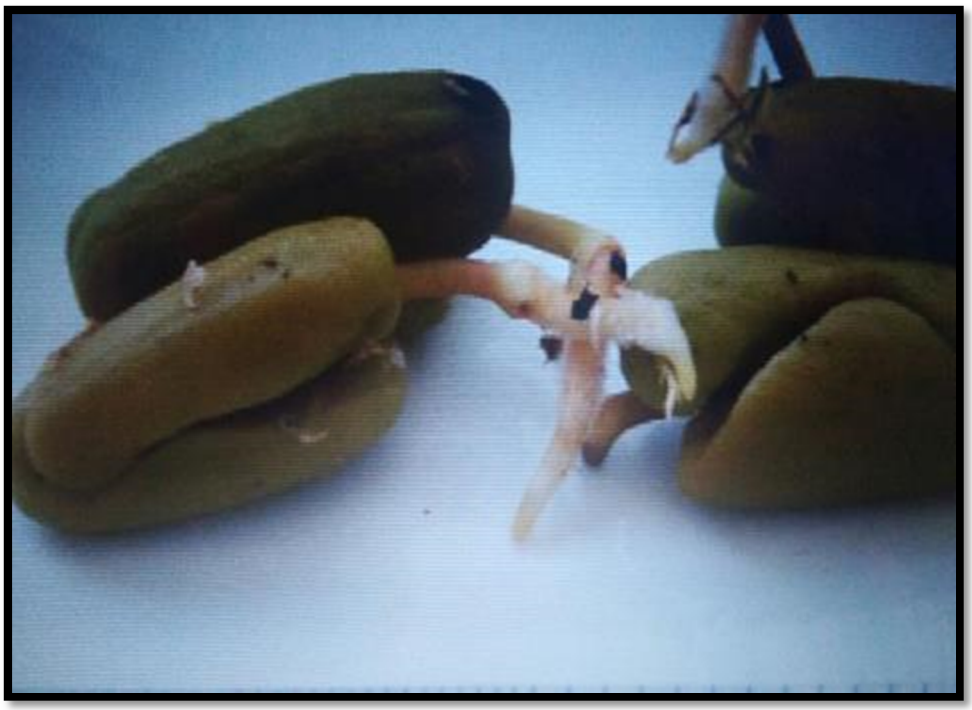


PLATE 7

ROOT OF S.aromaticum

lobes are fleshy,triangular,slightly incured.The four petals are imbricate,tinged red,rounded.

Colour	:	Greenish,turning pink at the time of maturity
Stamens	:	Stamens are very numerous,appearinggrouped in four masses.
Anther	:	Pale yellow,ovate,opening longitudinally wit small pale brown in colour

FRUITS(PLATE 5)

Shape	:	Oblong,fleshy, drupes
Size	:	The friuts are 2-3mm thick
Colour	:	Reddish purple in colour

SEEDS (PLATE 6)

Shape	:	Oblong,soft,grooved on one side,
Size	:	1.5 cm long

ROOT (PLATE 7)

The seedlings produces a pronounced tap root which remains relatively Short and is fairly quickly replaced by two or three primary sinkers which develop from it.

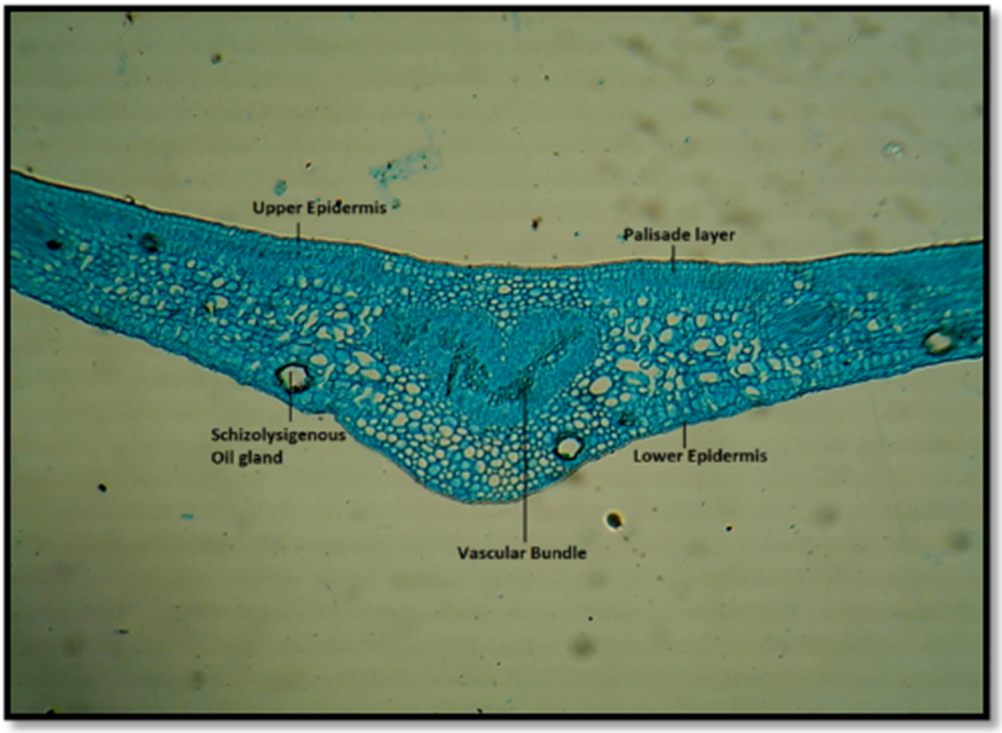
5.1.2 MICROSCOPY OF THE LEAF

LEAF MIDRIB (PLATE 8,9)

T.S of Midrib grooved from above erect downward flat on the adaxial side, And convexity on the adaxial side.

PLATE 8

T.S OF LEAF THROUGH MIDRIB [MICROTOME SECTION]



T.S OF MIDRIB [HAND SECTION]

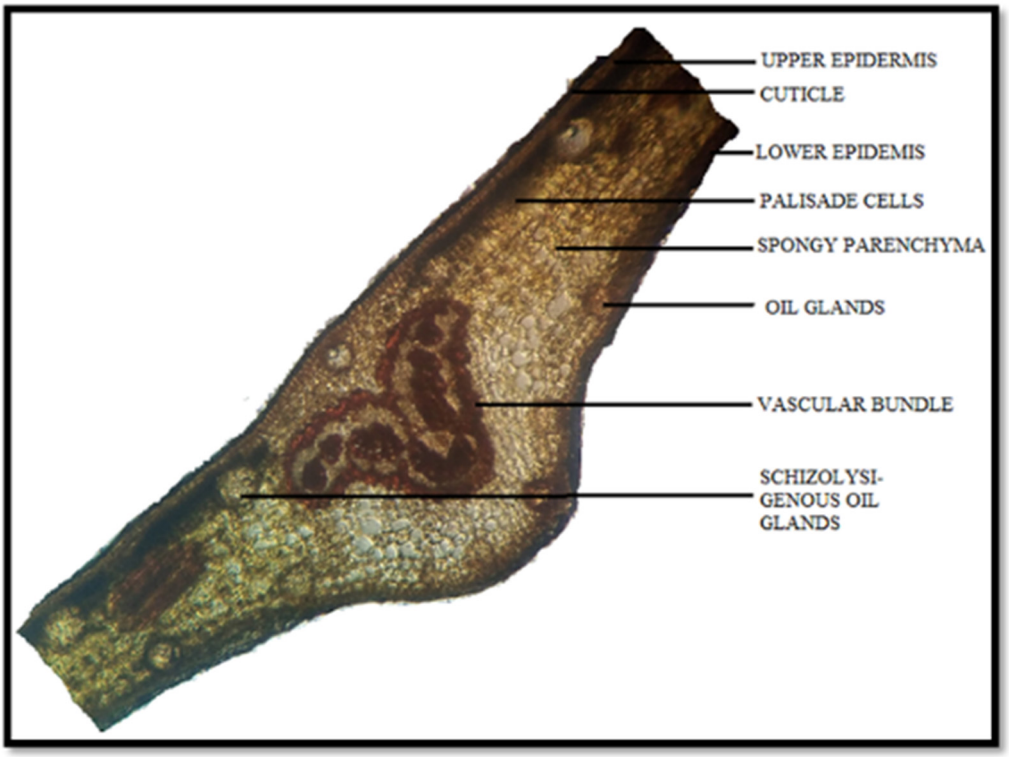
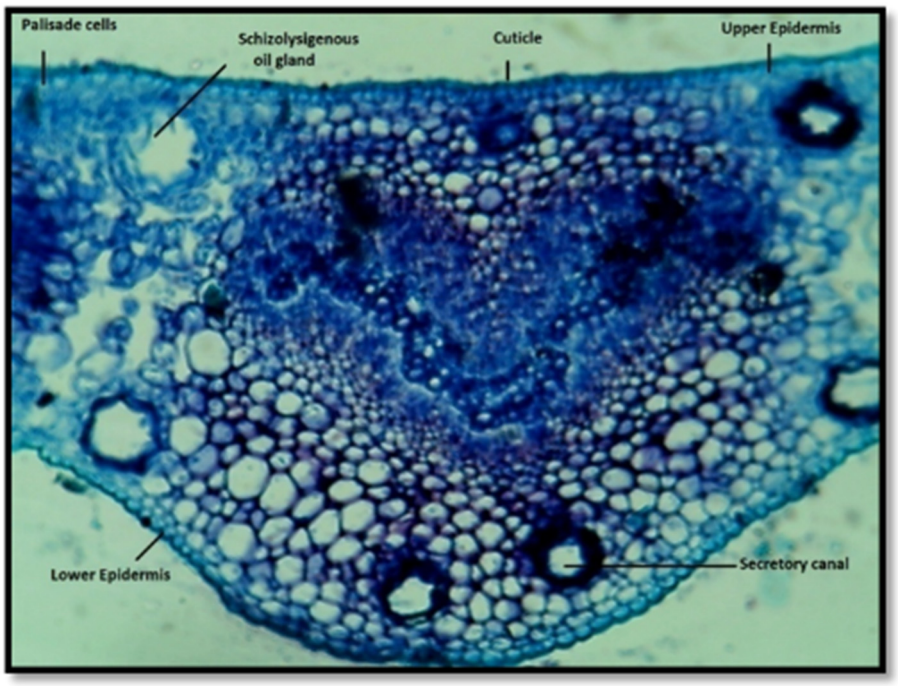


PLATE 9

MIDRIB ENLARGED [MICROTOME SECTION]



A PORTION OFLAMINA ENLARGED [MICROTOME SECTION]

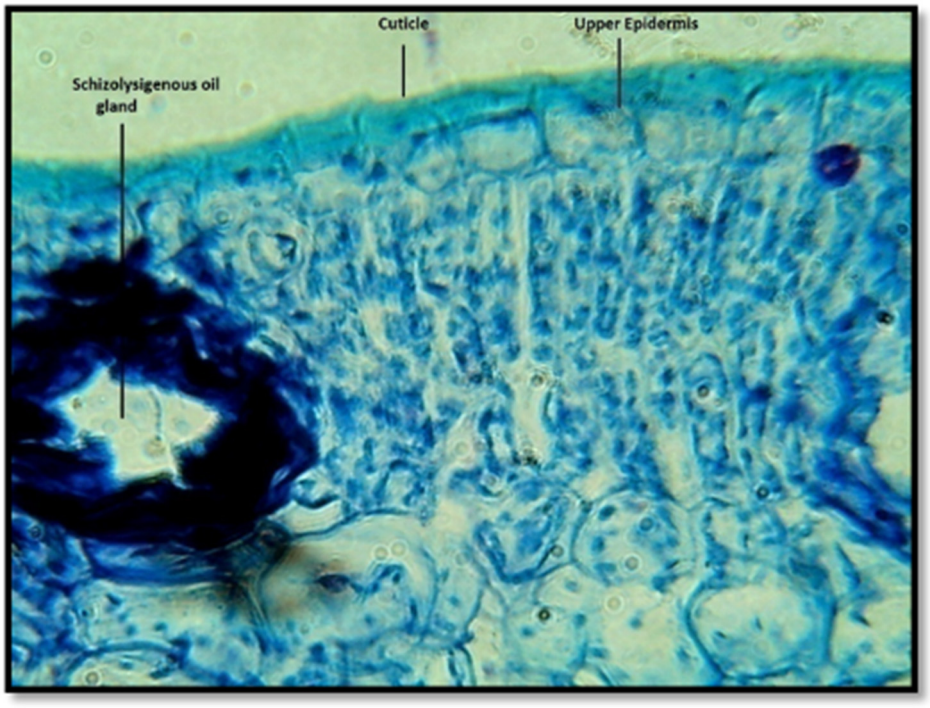
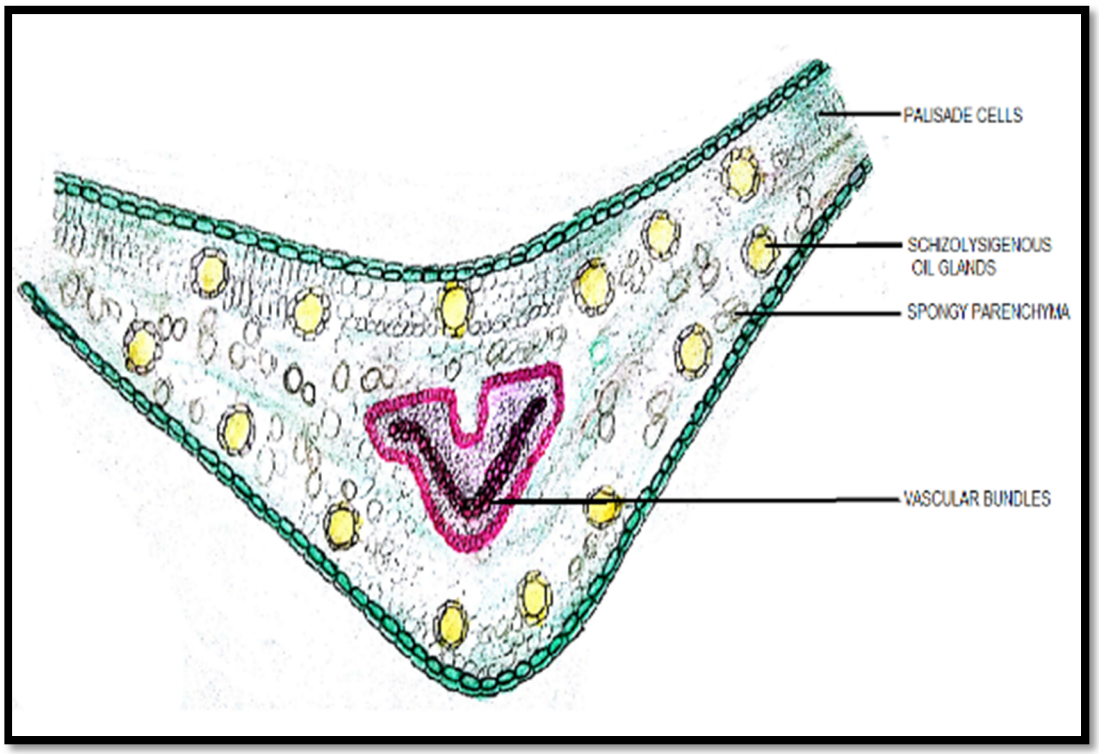


FIGURE 2

T S OF LEAF OF *S.aromaticum* THROUGH MIDRIB

[HAND DIAGRAM]



EPIDERMIS :(PLATE 10)

It is made up small rectangular thick wall uniseriatecells, covered by a thick cuticle. In surface view polygonal in shape.

STOMATA:

Amphistomatic,ranunculaceous few in upper and more in lower epidermis.

LAMINA (PLATE 10, 11):

Transverse section of lamina shows an upper epidermis with a cuticle. Stomata are present on both the epidermis followed by two layer of palisade cells containing chloroplasts. The spongy mesophyll is made up of 2 - 4 layers of cells and seen in between the palisade and lower epidermis cells. Some of the cells of the spongy parenchyma contain oil contents as secretary cavities developed initially schizogenous but ultimately lysigenous at maturity with a breakdown of all the secretary cells within the gland lumen. Oil cavities located close to both surfaces, glandular and lined with epithelial like cells. Cells containing tannin are very common.

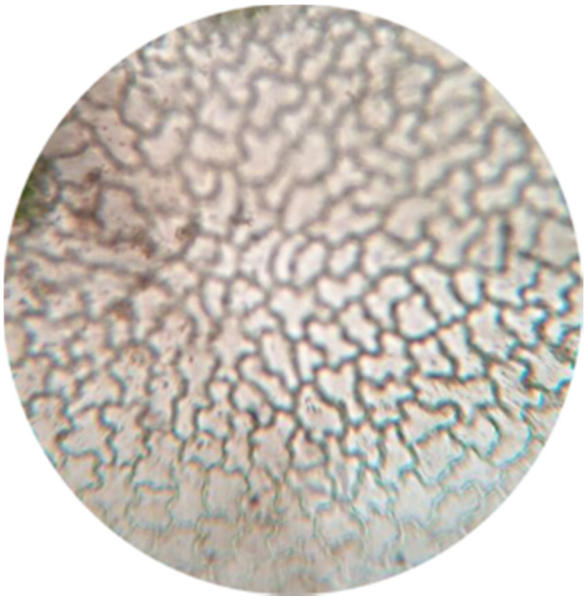
VASCULAR BUNDLES (PLATE 8,9 FIG 2)

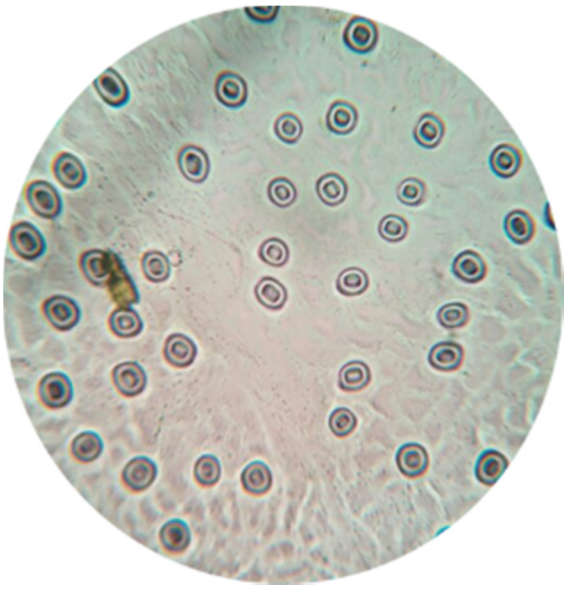
The vascular system consists of a U shaped or almost closed circle of separate collateral vascular bundles. Small vascular bundles are scattered towards the peripheral region. Each vascular bundle is encircled by a single row of bundle sheath.

PLATE 10

STOMATA (RANUNCULACEOUS)

UPPER EPIDERMIS **LOWER EPIDERMIS**





VENATION PATTERN OF *S.aromaticum*

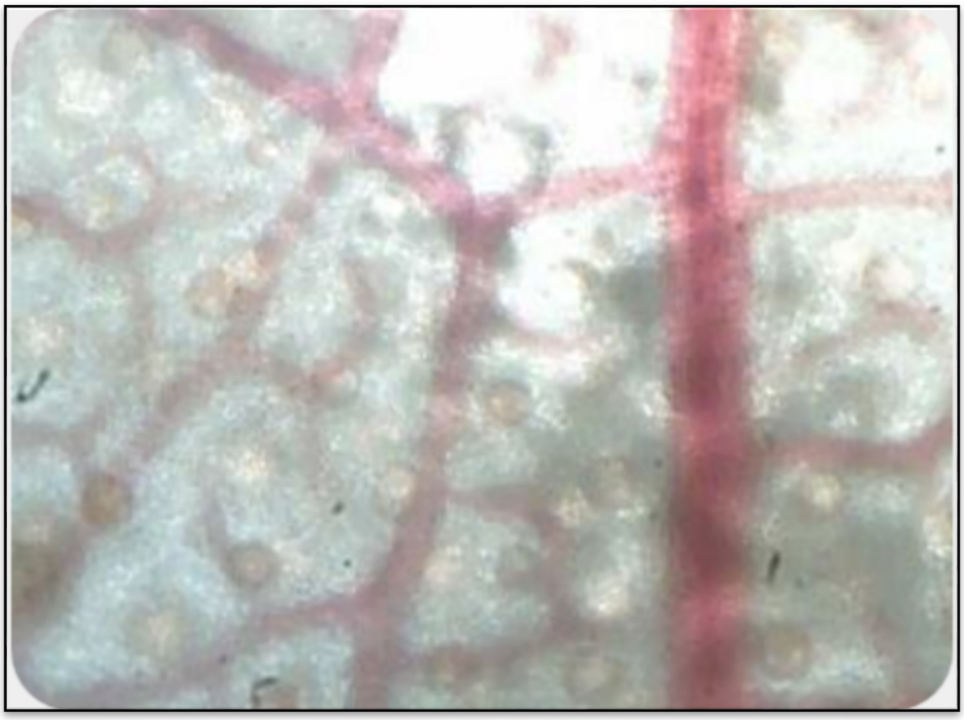
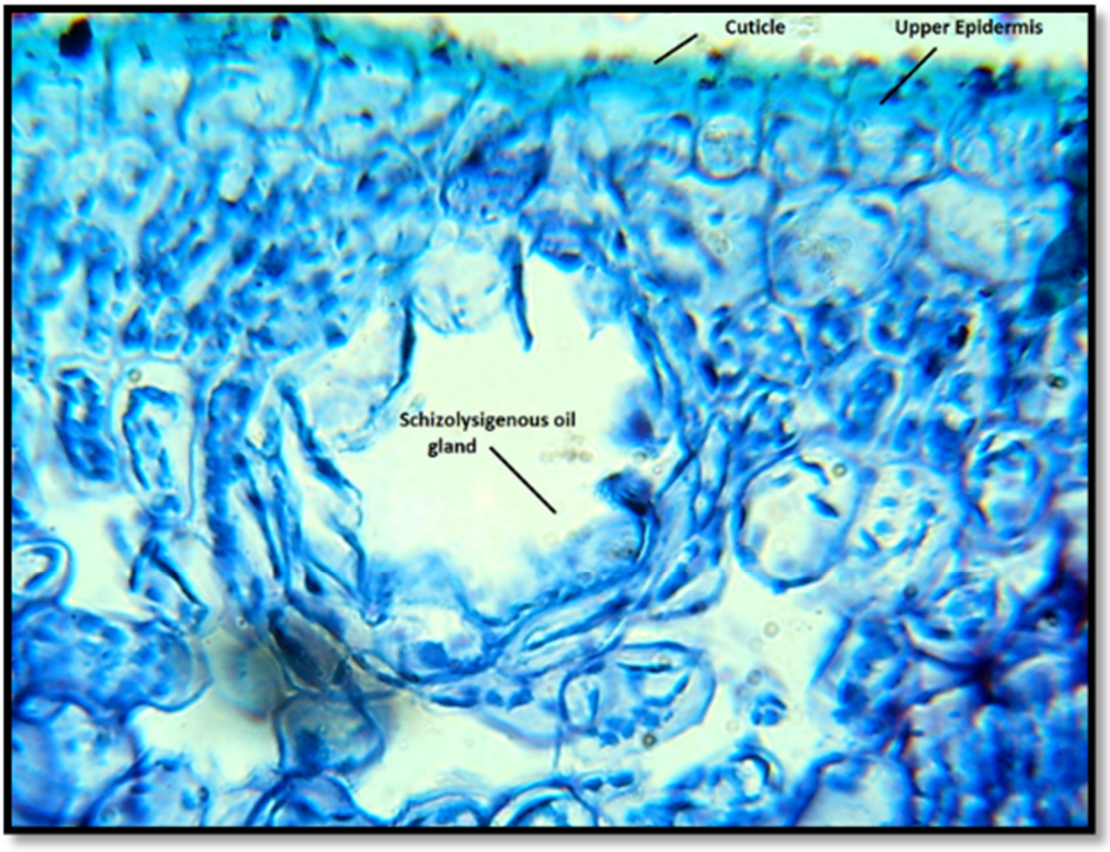


PLATE 11

SCHIZOLYSIGENOUS OIL GLAND ENLARGED

[MICROTOME SECTION]



GROUND TISSUE:

The ground tissue is made up of parenchymatous thin walled oval to circular, closely arranged cells with small intercellular spaces.

VENATION PATTERN (PLATE 13):

Distinct vein islets are formed by the secondary and tertiary veins. Vein islets are small and clearly seen, some of the vein terminations are forked.

T.S OF PETIOLE: (PLATE 12,13 FIG 30)

SHAPE:

Transverse section of petiole is oval in shape with a depression on the centre of the adaxial surface.

EPIDERMIS:

It has epidermal singlelayer of thick walled small rectangular cells with smooth thick cuticle

VASCULAR BUNDLE:

The Vascular system consists of single U shaped open circle vascular bundles. Ground tissue: It consists of parenchymatous cells. Large secreting cavities are present.

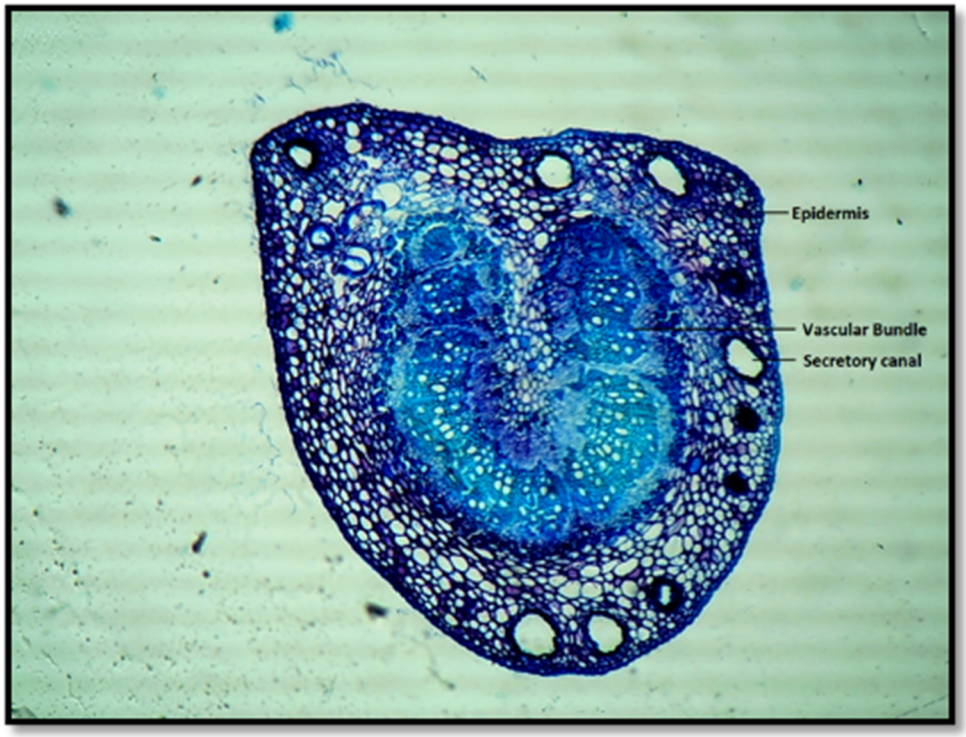
5.1.3 MICROSCOPICAL STUDY OF LEAF USING SCANNING ELECTRON MICROSCOPE:(PLATE 18)

SEM is a powerful magnification tool that utilizes focused beams of electrons to obtain information. The high resolution, three dimensional images produced by SEMS provide topographical, morphological & compositional information.

PLATE 12

T.S OF PETIOLE

(MICROTOME SECTION)



T.S OF PETIOLE [HAND SECTION]

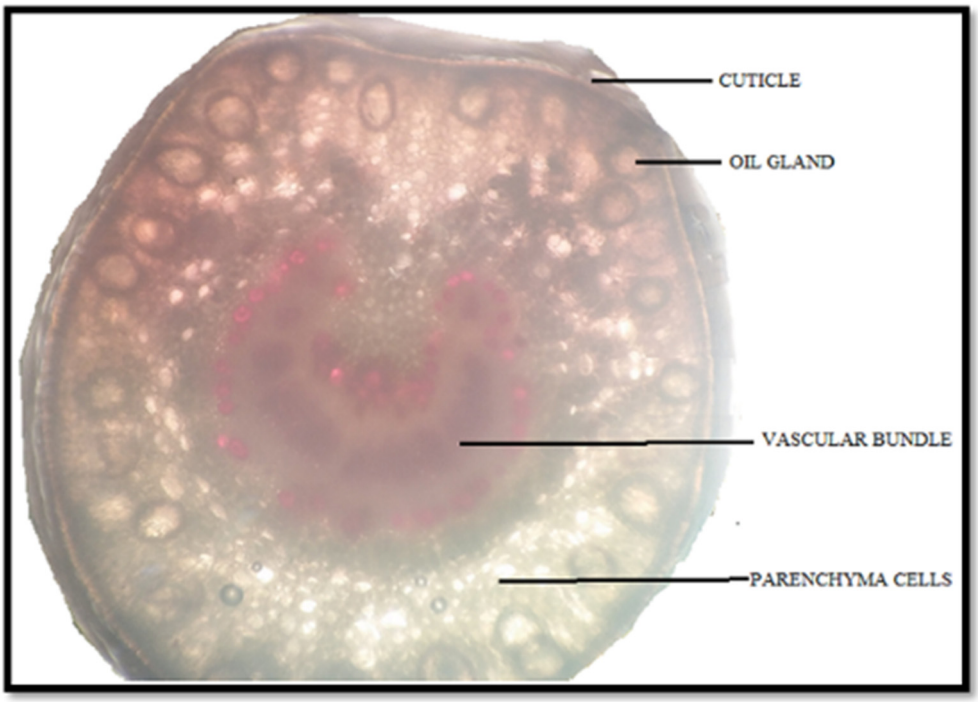


PLATE 13

T.S OF PETIOLE ENLARGED

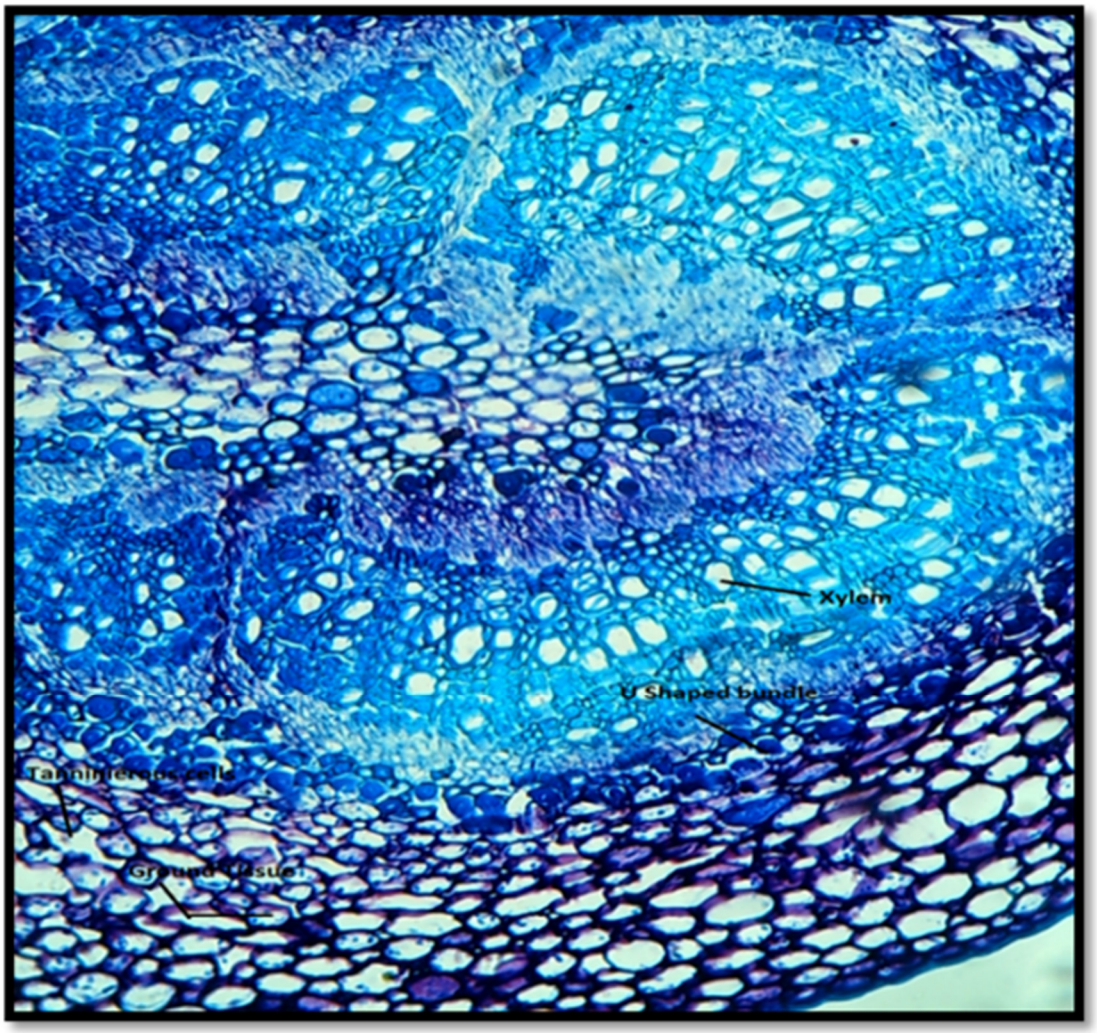
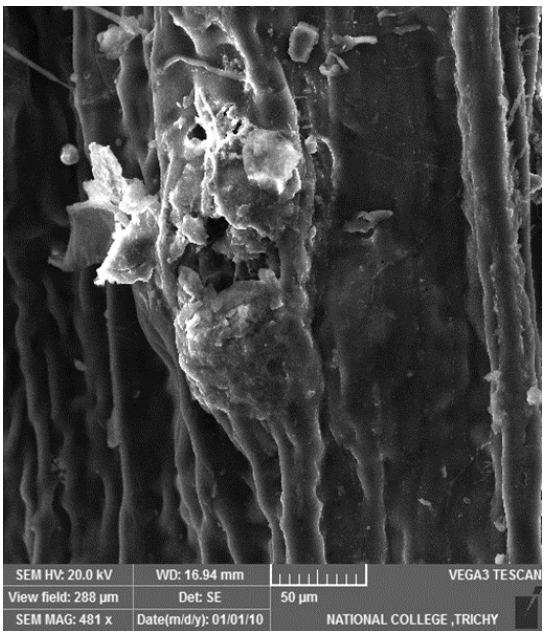
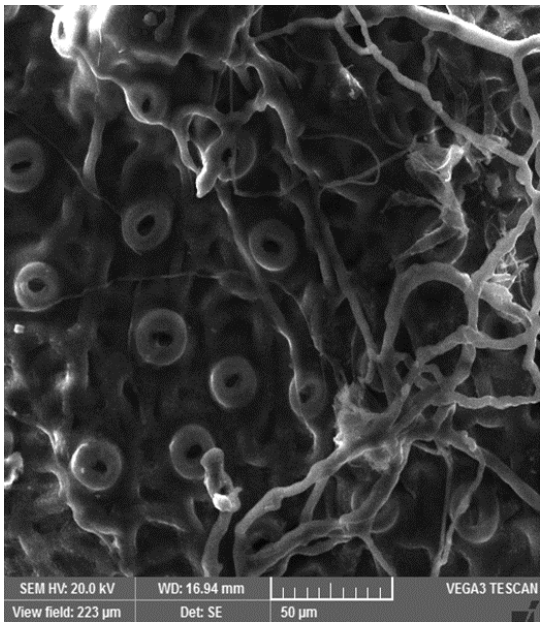


PLATE 14

SEM OF *S. aromaticum*





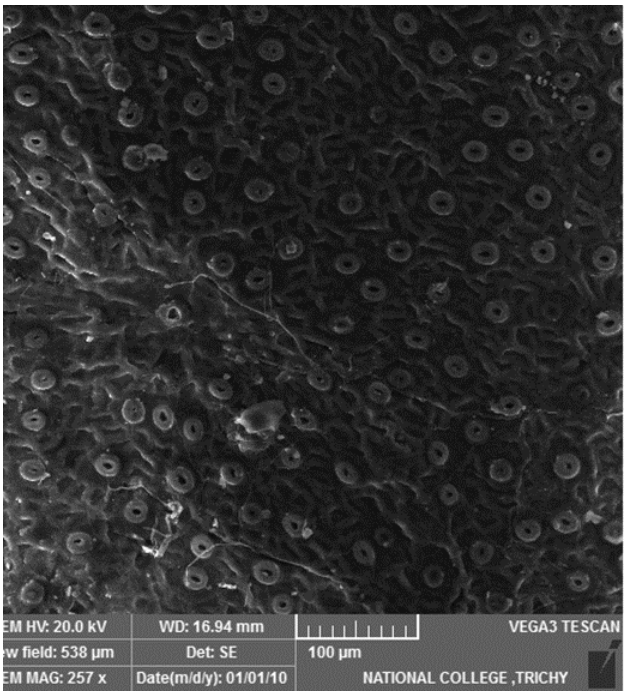
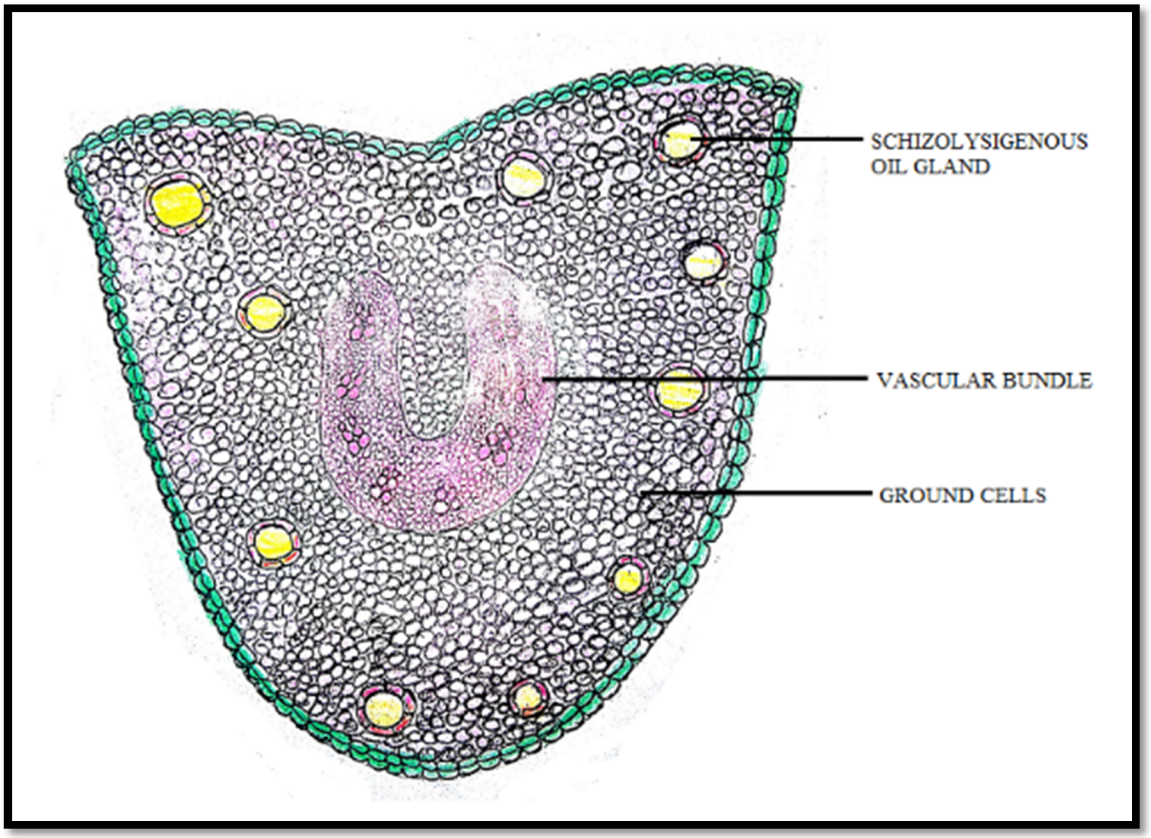


FIGURE 3

T S OF PETIOLE [HAND DIAGRAM]



SEM study of leaf provides detailed surface information. Adaxial and abaxial epidermal surface is smooth with no ornamentation. Trichomes are absent. Stomata are confined to the abaxial surface only.

5.1.4 POWDER MICROSCOPY

ORGANOLEPTIC CHARACTERS

- Nature : Coarse
- Colour : Dark green.
- Odour : Characteristic
- Taste : Aromatic
- Pressed in between two filter paper : No oil mark on the paper.

We have observed the following microscopical cell structures,

- Secretory cavity
- Ranunculaceous stomata
- Xylem
- Phloem with companion cells
- Fibres
- Parnenchyma cells
- Schyzolysigenous cells

5.1.5 MICROSCOPIC SCHEDULES

As per the methods described in materials and methhods, microscopic schedules were carried out and the results tabulated from the Tables 1- 4

FIGURE - 4

POWDER MICROSCOPICAL CHARACTER OF *S.aromaticum*

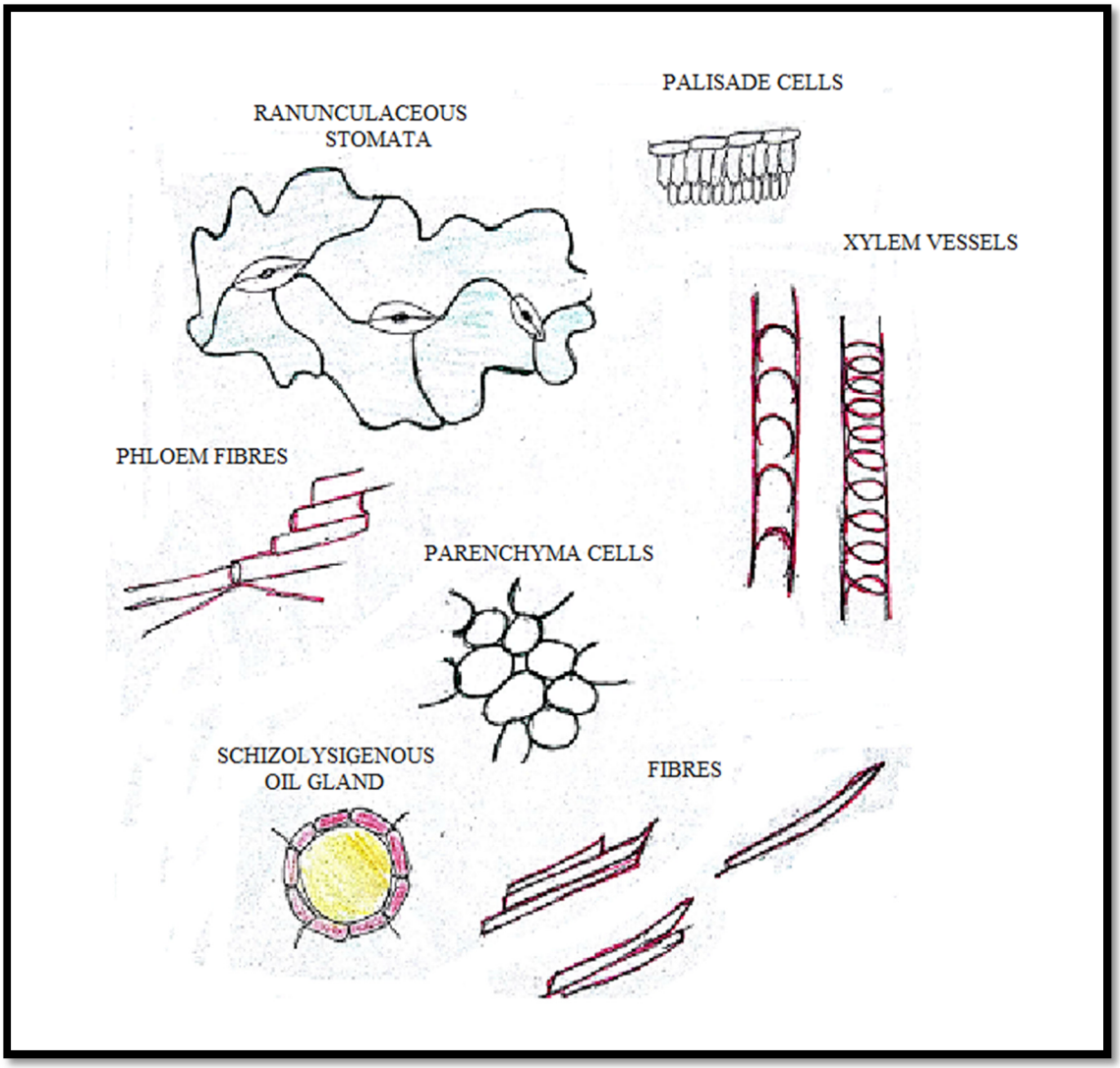


Table – 1

VEIN ISLET AND VEIN TERMINATION NUMBER

OF*Syzygium aromaticum* LEAVES

OBSERVATION NUMBER	VEIN ISLET NUMBER	VEIN TERMINATION NUMBER
1	8	6
2	7	6
3	8	6
4	7	6
5	6	5
6	7	6
7	6	4
8	8	7
9	8	5
10	7	6
Minimum	**6**	**4**
Average	**7.2**	**5.7**
Maximum	**8**	**7**

Table 2

STOMATAL NUMBER OF *S.aromaticum* LEAVES

OBSERVATION NUMBER	UPPER EPIDERMIS	LOWER EPIDERMIS
1	5	9
2	4	8
3	3	6
4	4	8
5	3	8
6	4	8
7	5	9
8	5	8
9	3	8
10	5	7
Minimum	**3**	**6**
Average	**4.1**	**7.9**
Maximum	**5**	**9**

Table – 3

STOMATAL INDEX OF *S.aromaticum* LEAF

OBSERVATION NUMBER	UPPER EPIDERMIS	LOWER EPIDERMIS
1	24	42
2	26	40
3	28	35
4	26	37
5	23	39
6	26	48
7	24	44
8	24	45
9	26	44
10	28	46
Minimum	**23**	**35**
Average	**25.5**	**42**
Maximum	**28**	**48**

Table – 4

PALISADE RATIO OF *S.aromaticum* LEAF

S.NO	PALISADE RATIO
1	4
2	3
3	3
4	5
5	4
6	3
7	4
8	3
9	4
10	4
Minimum	**3**
Average	**3.7**
Maximum	**5**

5.1.6 PHYSICO CHEMICAL PARAMETERS

As per the methods described in materials and methods, physicochemical parameters were carried and the results were as follows.

Table – 5

ASH VALUES OF THE *S.aromatium* LEAVES

OBSERVATION NUMBER	TOTAL ASH (%)	ACID INSOLUBLE ASH (%)	WATER SOLUBLE ASH (%)
1	7.5	0.7	-
2	7.9	0.6	-
3	7.5	0.9	-
4	7.06	0.6	-
5	7.14	1.1	-
6	7.5	-	1
7	7.18	-	1.12
8	7.03	-	1.05
9	7.05	-	1.02
10	7	-	1.06

ASH	MINIMUM	AVERAGE	MAXIMUM
TOTAL ASH	7.03	7.286	7.9
ACID INSOLUBLE ASH	0.6	0.78	1.1
WATER SOLUBLE ASH	1	1.05	1.12

Table – 6

LOSS ON DRYING OF *S.aromaticum* LEAVES

OBSERVATION NUMBER	LOD %w/w
1	9.12
2	8.55
3	8.51
4	7.5
5	8.78
Minimum	**7.50**
Average	**8.49**
Maximum	**9.12**

Table – 7

EXTRACTIVE VALUES FOR *S.aromaticum* LEAVES

SOLVENTS	EXTRACTIVE VALUE (%)
Petroleum ether	5.2
Ethanol	9.8
Water	10.6

5.2. PHYTOCHEMICAL STUDIES

5.2.1. PRELIMINARY PHYTOCHEMICAL SCREENING:

QUALITATIVE PHYTOCHEMICAL TEST

Preliminary phytochemical screening of the powdered mature leaves were carried out and the results are as follows (Table 8)

TEST FOR ALKALOIDS

Mayer's test	:	Cream precipitate shows the **presence** of alkaloids
Dragendorff's test	:	Reddish brown precipitate shows the **presence** of alkaloids
Hager's test	:	Yellow precipitate shows the **presence** of alkaloids

TEST FOR CARBOHYDRATES

Molish's test	:	Appearance of purple colour shows the **presence** of carbohydrates.
Fehling's test	:	Formation of reddish brown precipitate shows the **presence** of free reducing sugars.
Benedict's test	:	Formation of reddish brown precipitate shows the **presence** of free reducing sugars.

TEST FOR GLYCOSIDES

Keller killiani's test	:	No reddish brown colour ring at the junction shows The **Absence** of cardiac glycosides.

Borntrager's test : No Appearance of pink colour in ammonicallayer shows the **Absence** of anthraquinone glycosides

Modified Borntrager's test : No Appearance of pink colour in ammonical layer shows the **absence** of anthraquinone glycosides

TEST FOR PHYTO STEROL

Salkowski's test : Appearance of red colour in lower layer shows the **Presence** of sterol

Liebermann – Burchard's test : Brown ring at the junction of two layers and green colour in the upper layer shows the **Presence** of sterols

TEST FOR SAPONINS : Frothing occurs indicates the **presence** of Saponins

TEST FOR TANNINS

Ferric chloride test : Appearance of bluish black colour shows the**presence**of tannins

Gold beater's skin : Appearance of brown colour shows the **presence** of tannins

TEST FOR PROTEINS AND FREE AMINOACIDS

Millon's test	:	Appearance of red colour on heating shows the **presence** of proteins
Biuret test	:	Appearance of violet colour shows the **presence** of proteins
Ninhydrin test	:	Formation of violet colour shows the **presence** of amino acids
TEST FOR MUCILAGE	:	No appearance of reddish pink colour shows the **presence** of mucilage
TEST FOR TERPENOIDS	:	Appearance of pink colour shows the **presence** of terpenoids

TEST FOR FLAVONOIDS

Shinoda test	:	Purple colour shows the **presence** of flavonoids
Alkaline reagent test	:	Yellow - orange colour shows the **presence** of flavonoids
Acid test	:	Yellow – orange colour shows the **presence** of flavonoids
Zinc hydrochloride	:	Red colour shows the **presence** of flavonoid
TEST FOR VOLATILE OIL	:	Volatile oil obtained shows the **presence** of volatile oil

TEST FOR FIXED OIL : No translucent greasy spot shows the absence of fixed oils

Table – 8

PRELIMINARY PHYTOCHEMICAL SCREENING OFLEAVES OF *S.aromaticum*

S.NO	TEST	OBSERVATION
I.	**ALKALOIDS**	
	Mayer's reagent	+
	Dragendorff's reagent	+
	Hager's reagent	+
II.	**CARBOHYDRATES**	
	Molisch's test	+
	Fehling's test	+
	Benedict's test	+
III.	**GLYCOSIDES**	
	Anthroquinone glycosides	-
	Borntrager's test	-
	Modified Borndrager's test	-
	Cardiac glycosides	
	Keller Killiani test	-
	Raymond test	-
	Legal test	-
IV.	**STEROLS**	
	Salkowski test	+
	LibermanBurchard's test	+
V.	**SAPONINS**	+
VI.	**TANNINS**	
	Ferric chloride	+
	Gold Beater's skin test	+
VII.	**PROTEINS AND FREE AMINO ACIDS**	
	Millon's test	+
	Biuret test	+

	Ninhydrin test	+
VIII.	**MUCILAGE**	+
IX.	**TERPENOIDS**	+
X.	**FLAVONOIDS**	
	Shinoda test	+
	Alkali test	+
	Acid test	+
	Zn/Hcl test	+
XI.	**VOLATILE OIL**	+
XII.	**FIXED OIL**	-

5.2.2 FLUORESCENCE ANALYSIS

The fluorescence analysis of the leaf powder of ***S.aromaticum*** was studied. The results were as follows (Table -9**)**

Table -9

FLUORESCENCE ANALYSIS

REAGENT	OBSERVATION
Powder as such	Dark Green
Powder + 50% Hydrochloric acid	Yellowish Green
Powder + 50% Nitric acid	Light brown
Powder + Petroleum ether	Light orange
Powder + 50% Sulphuric acid	Dark brown
Powder + 1N NaOH in water	Dark green
Powder + 1N NaOH in methanol	Dark Green
Powder +5% Ferric chloride solution	Greenish brown
Powder + Picric acid	Flourescence green
Powder + Chloroform	Green
Powder + 5% Iodine solution	Green
Powder + (HNO3 + NH3)	Green

5.2.3 ESTIMATION OF FLAVONOID CONTENT

Flavonoid content of **extract** in terms of quercetin by aluminium chloride was found to be **23.1mg/g**.

5.2.4 ESTIMATION OF TOTAL PHENOLIC CONTENT

Total phenolic content ofextractin terms of gallicacid was found to be **3.7mg/g**.

5.2.5 DETERMINATION OF TRACE ELEMENTS IN THE LEAF OF *S.aromaticum* BY ENERGY DISPERSIVE X-RAY SPECTROMETER (EDS)ENERGY DISPERSIVE X-RAY SPECTRUM FOR *S.aromaticum* LEAVES

Estimation of the elements like Cl, K, C, Ca, N, O, Fe, Mg, Al, and Si showed the following mg weight percentage and atomic percentage.

FIGURE – 5

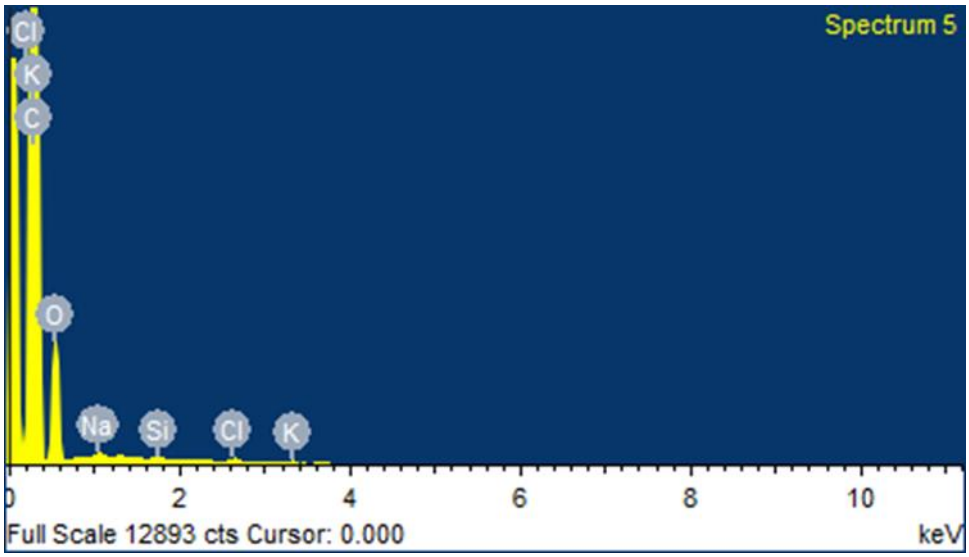


Table-10

***S.aromaticum* LEAVES ELEMENTS WEIGHT & ATOMIC PERCENTAGE**

Element	Weight %	Atomic %
C K	78.45	82.99
O K	21.23	16.86
Na K	0.12	0.07
Si K	0.05	0.02
Cl K	0.08	0.03
K K	0.06	0.02
Totals	100.00	

5.2.6 PHYSICO CHEMICAL EVALUATION OF ISOLATED VOLATILE OIL

The Result of Phyico Chemical Analysis ,GC- MS Analysis Were As Follows,

1.Percentage Of Oil Obtained:2.5%

2.Colour:Pale Yellow

3.Odour:Aromatic

4.Taste:Pungent

5.CharacteristicFeel:Greasy

6.Solubility:Soluble In Petroleum Ether,Toluene,Chloroform,Ethanol.

ImmisibleWith Water

7.Refractive Index:1.533-1.359

8.Specific Gravity:1.032-1.067

9.Optical Rotation:-$0^0$50” to - 1^o53”

5.2.7.IDENTIFICATION OF COMPOUNDS PRESENT IN THE VOLATILE OIL OF LEAVES BY GC-MS ANALYSIS

The GC-MS analyscis of the isolated V.O indicated the presence of following constituents bycomparing with the instrument library. β-caryophyllene, Globulol α -pinene, α -terpinene, β- bisabolene, Eugenol,Limonene,caryophylene oxide, cadinol, m.Cymene.

FIGURE – 6- GC-MS PROFILE OF THE V O OF THE *S.aromaticum* LEAVES

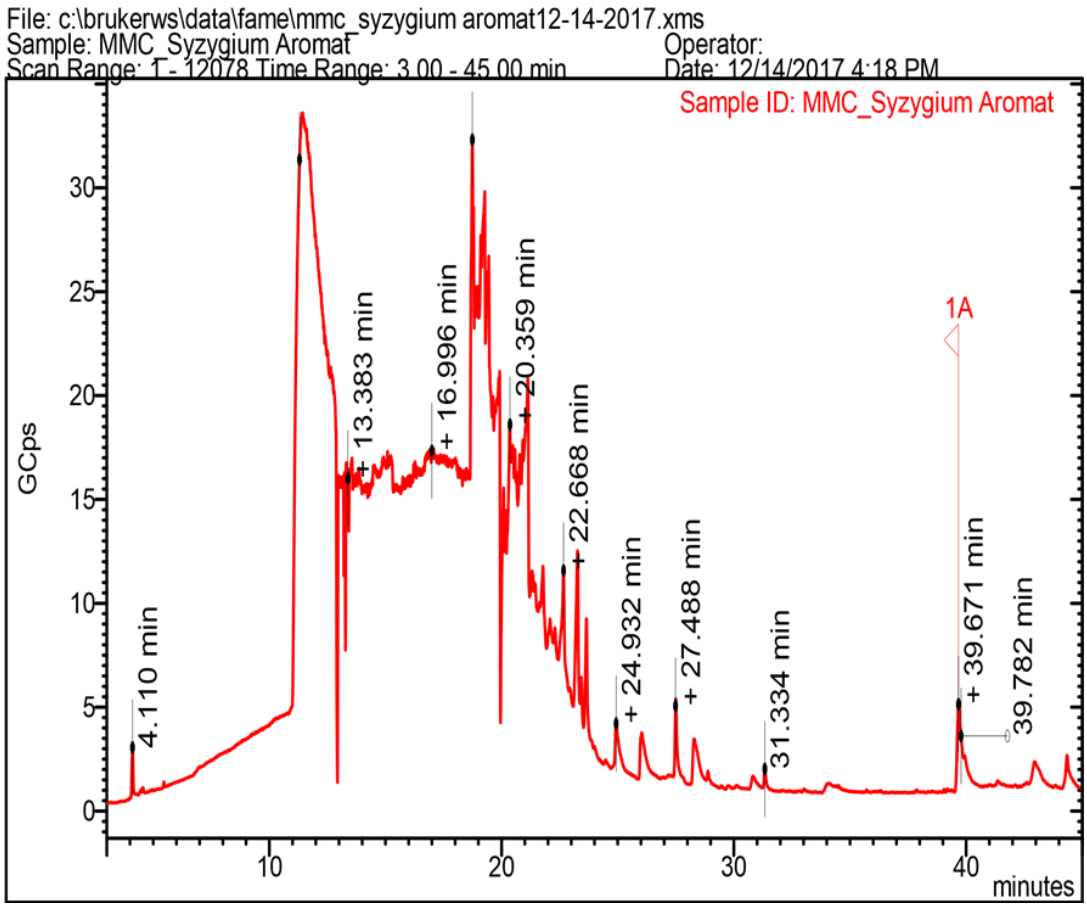
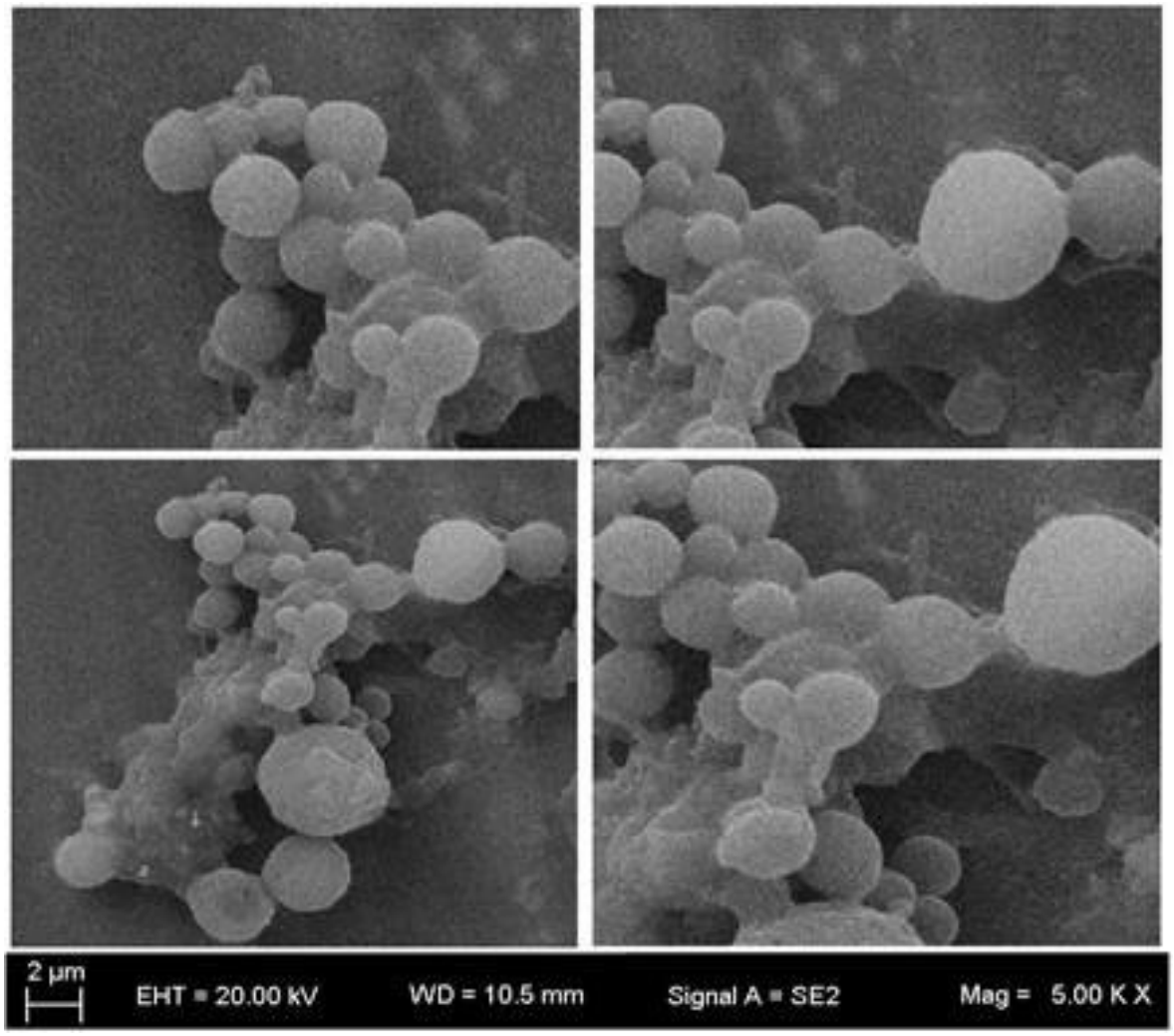


FIGURE -6 A

SEM IMAGES OF NANOSPHERES OF S.*aromaticum* LEAF VOLATILE OIL



Preparation of nanospheres of volatile oil of the leaves of *S.aromaticum* (SALVONS):

Encapsulation of VO using 1:1 (w/w) ratio EC/MC as the polymeric shell material was performed, resulted in the formation of milky white aqueous suspension. Analysis of the suspension by SEM revealed the presence of almost spherical particles with a dry size average diameter of 424± 102.6 nm. The process gave an EE of 77.5% with a loading level of 44.2% w/w. (Figure-6A)

5.3 PHARMACOLOGICAL STUDIES

5.3.1 Acute Toxicological Study Using Brine Shrimp Lethality Assay (BSLA) EFFECT OF VARIOUS CONC OF VOSAL ON *Artemia nauplii*

In continuation of our efforts to verify the safety of VO, we performed Brine shrimp lethality assay (BSLA) using free swimming hatched out *Artemianauplii* which based on the ability to kill laboratory cultured brine shrimp. It was observed that 100% of mortality above 800 ppm for VO. LC_{50} for VO was about 500ppm respectively in 24hrs. 100% mortality was observed at 3ppm for podophyllotoxin positive control.

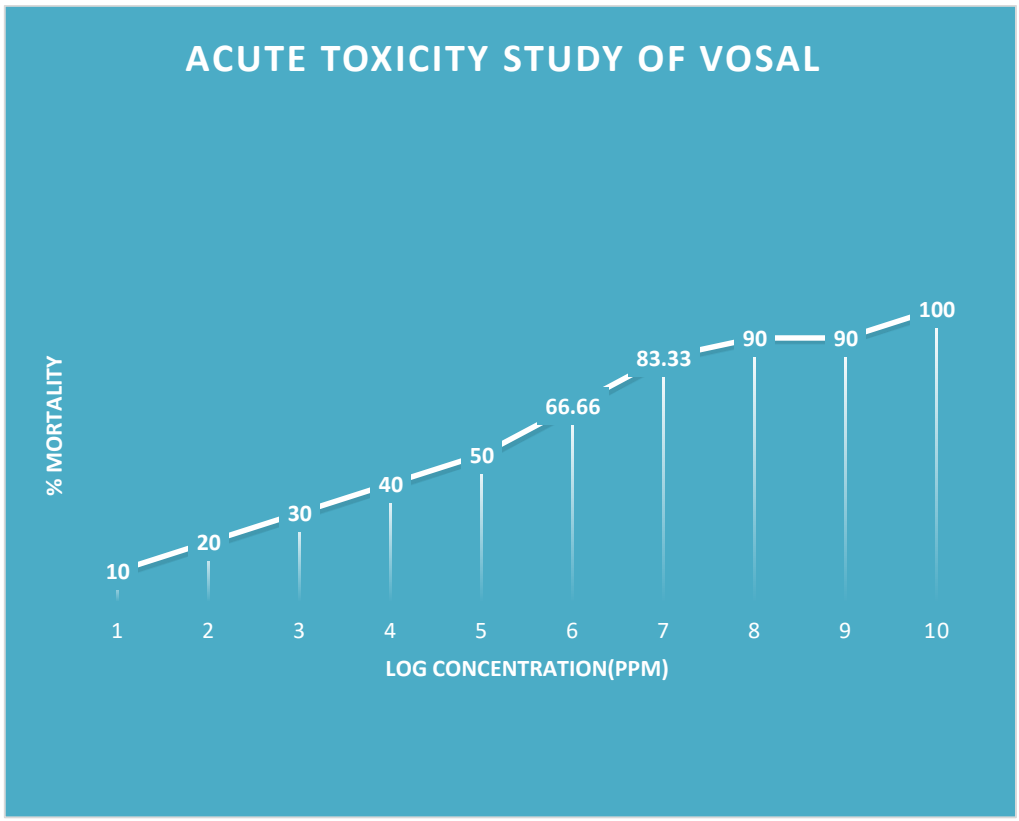
TABLE 11

ACUTE TOXICITY STUDY OF VOSAL (BSLA)

CONCENTRATION (PPM)	NUMBER OF LARVAE RELEASED	NUMBER OF LARVAE DEAD AFTER 24HRS	MORTALITY (%)	CORRECTED (%) MORTALITY USING ABBOT'S FORMULA
100	10	1	10	10
	10	1	10	
	10	1	10	
200	10	1	10	20
	10	2	20	
	10	3	30	
300	10	2	20	30
	10	3	30	
	10	4	40	
400	10	4	40	40
	10	4	40	
	10	4	40	
500	10	5	50	50
	10	5	50	
	10	5	50	
600	10	6	60	66.66
	10	7	70	
	10	7	70	
700	10	8	80	83.33
	10	8	80	
	10	9	90	
800	10	9	90	90
	10	9	90	
	10	9	90	
900	10	8	80	90
	10	9	90	
	10	10	100	
1000	10	10	100	100
	10	10	100	
	10	10	100	

FIGURE 7

ACUTE TOXICITY STUDY OF VOSAL



5.3 .2 EFFECT OF VOSAL ON MUTAGENESIS OF *Drosophila melanogaster*:

Morphological changes like eye colour, spots in the wings, wing hairs, changes in the length and width of the wing, wing shape, abdomen length and total body length were observed in the F1 generation in the VO and formalin treated group. (Table 13). There was no observable morphological changes in the test drug treated but change of eye colour in the formalin treated. (Plate 1)

TABLE-12

EFFECT ON MUTAGENESIS OF VOSAL OF*S.aromaticum* on *Drosophila melanogaster*

S. No	Morphology	Normal Flies	Std Formaldehyde Exposed Flies (0.5% V/V)	VO treated Flies (µl/ml)		
				1	2	4
1.	Eye Colour	Red	Dark pink	Red	Red	Red
2.	Wing Shape Spots Hairs Length Width	 Elliptical Present Present 100±1.1 42±1.2	 Elliptical Present Present 82±0.98 36±1.78	 Elliptical Present Present 95±1.41 44±0.87	 Elliptical Present Present 96±1.1 43±2.1	 Elliptical Present Present 99±1.3 42±2.4
3.	Abdomen Length µm	42±1.14	41±1.8	42±0.82	41±0.99	40±1.4
4.	Total Body Length µm	97.3±2.02	95.8±1.9	94.2±0.56	95.66±1.22	95.93±1.72

5.3.3.*In vivo* EFFECT OF SALVONS ON THE TRANSGENIG *Drosophila melanogaster* MUTANT WITH Aβ42 INDUCED NEURODEGENERATION.

5.3.4 EFFECT OF SALVONS ON LONGEVITY OF Aβ42 EXPRESSING *Drosophila*

In the present study neuroprotective effect was evaluated using *Drosophila* AD model. This lifespan experiments Aβ42*Drosophila* showed a complete reduction in lifespan between 30 -40 days. All treated groups showed significant improvement of survival. 5µl/g showed maximum lifespan increase equivalent to the standard drug donepezil (Table 13)

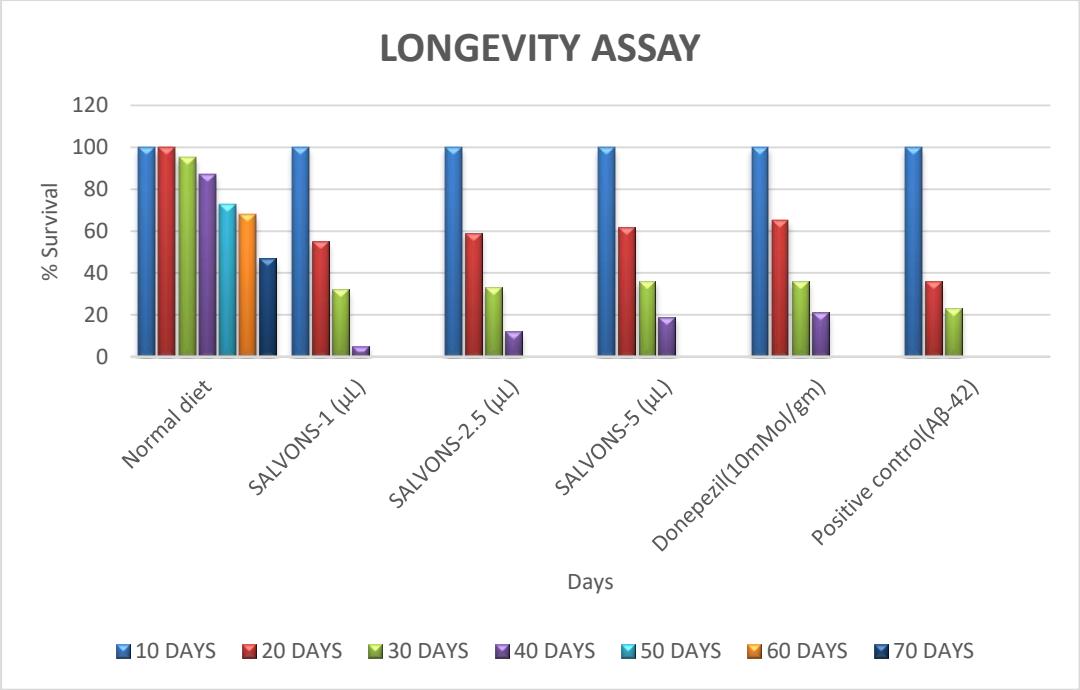
TABLE 13

LONGEVITY ASSAY

Name of the substance	Conc/gm	% survival						
		Days						
		10	20	30	40	50	60	70
SALVONS	1(µl)	100	55	32	5	0	0	0
	2.5(µl)	100	59	33	12	0	0	0
	5(µl)	100	62	36	19	0	0	0
Donepezil	10mMol/gm	100	65	36	21	0	0	0
POSITIVE Control-Aβ 42	1%DMSO	100	36	23	0	0	0	0
Normal control	Normal Diet	100	100	95	87	73	68	47

FIGURE 8

LONGEVITY ASSAY



5.3.5 EFFECT OFSALVONS ON LOCOMOTOR FUNCTION BY CLIMBING ASSAY OF Aβ42 EXPRESSING *Drosophila*:

For locomotor abilities determination Aβ42 expressing *Drosophila* showed significant impaired locomotion from the age of day 9 onwards. SALVONS treated flies showed an improvement in locomotor activity dose dependently. 5µl/g conc showed improvement in the locomotion equivalent to the std drug donepezil. (Table-14, Fig-8)

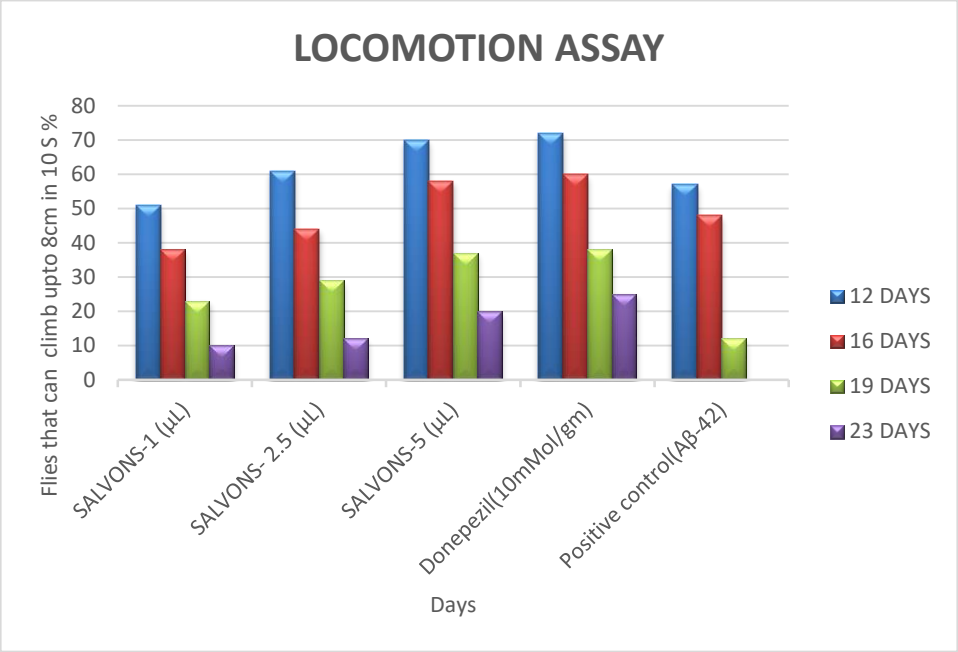
TABLE 14

LOCOMOTION ASSAY

EXTRACT	CONC/gm	DAYS			
		12	**16**	**19**	**23**
SALVONS	1(µl)	51	38	23	10
	2.5(µl)	61	44	29	12
	5(µl)	70	58	37	20
Aβ42 (+ve control)	1%DMSO	57	48	12	0
Donepezil	10mMol/gm	72	60	38	25

FIGURE 9

LOCOMOTION ASSAY



5.3.6.PSEUDOPUPIL TECHNIQUE TO VISUALIZE THE RETINO RHABDOMERES IN ADULT EYES:

In this study the effect of Aβ42 expressing *Drosophila* on degeneration of retinal tissue which were mainly neurons. Aβ42 expressing *Drosophila* contained significantly more degenerating rhabdomeres compared with the normal fly. The number of degenerated rhabdomeres was 2±0.258. The treated group has significantly rescued rhabdomeres in each ommatidium with increase in number of count which reflected a preventive effect of the SALVONS on neurodegeneration. This effect was comparable to the standard drug donepezil

TABLE 16

EXPERIMENT		RHABDOMERE COUNT
Normal Control		7
Aβ-42 expression		2±0.458
Donepezil		4.45±0.89
	1µl	2.55±0.39
SALVONS treated	2.5 µl	3.78±0.78
	5 µl	4.1±0.16

FIGURE 10

PSEUDOPUPIL ASSAY

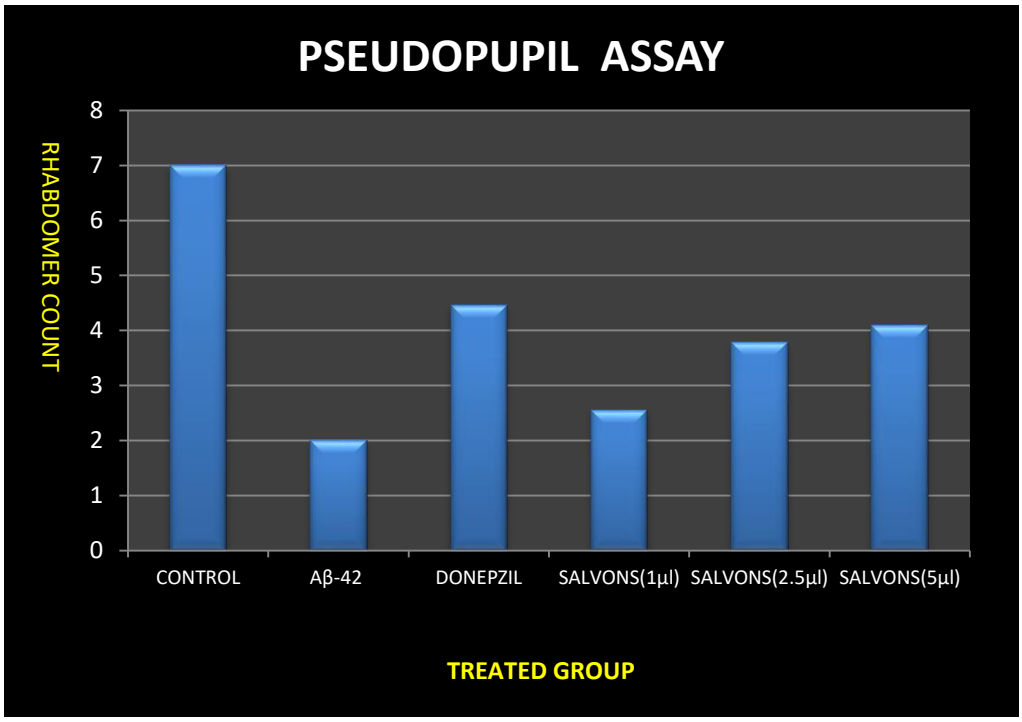
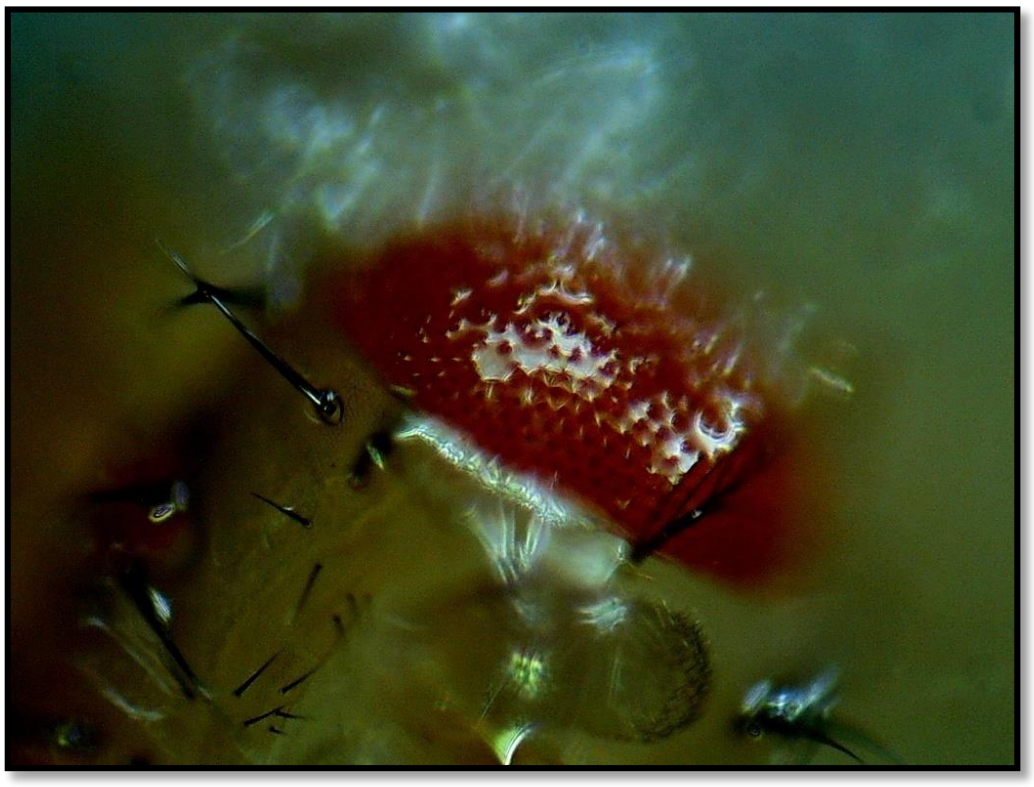


PLATE 13: PSEUDOPUPIL SHOWING RHABDOMERES

CONTROL

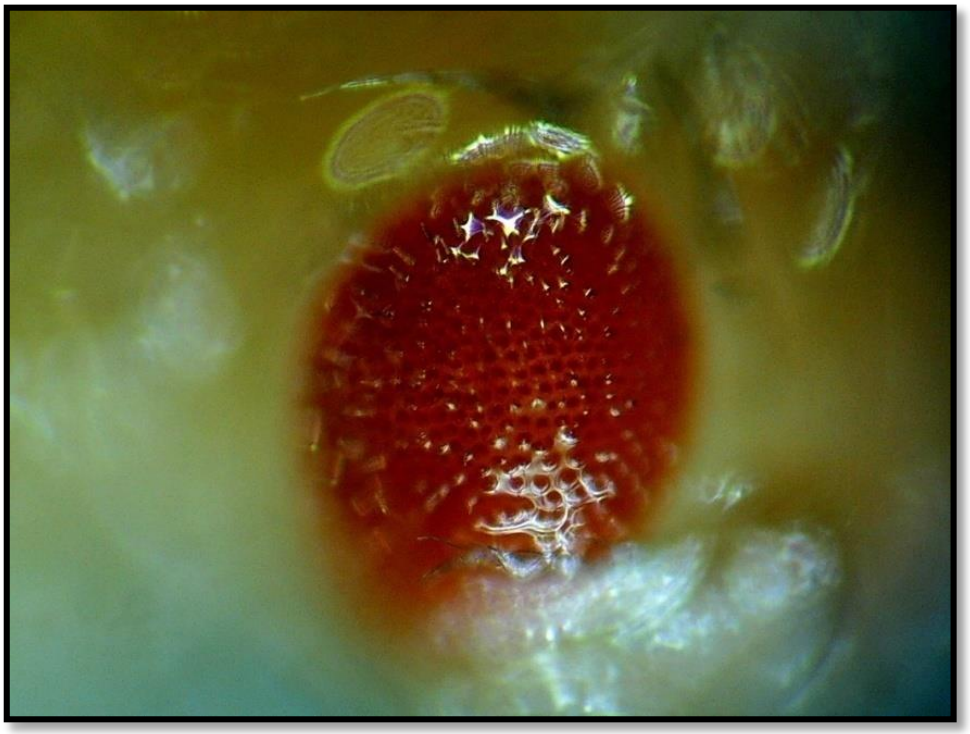


Aβ 42 CONTROL

PSEUDOPUPIL SHOWING RHABDOMERES

STANDARD DRUG TREATED

SALVONS TREATED



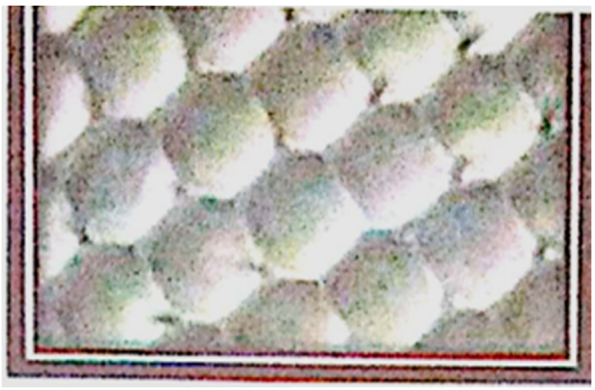
5.3.7. EVALUVATION OF SURFACE ORGANIZATION OF OMMATIDIA IN ADULT EYES BY NAIL – POLISH IMPRINTS AND SEM:

To examine any disruption in the ordered arrays of the ommatidia in adult eyes a novel, simple and inexpensive method that provides high quality images, comparable to those obtained by SEM, was adopted by using the nail polish technique. The peeled off layer being the exact replica of the eye showed the surface morphology. It was observed that there was a disruption in the regular array of the ommatidia in the Aβ42 expressing *Drosophila*. But the treated group and the standard drug treated group showed improvement of the surface with reduction in disruption. (Plate 14)

PLATE 15

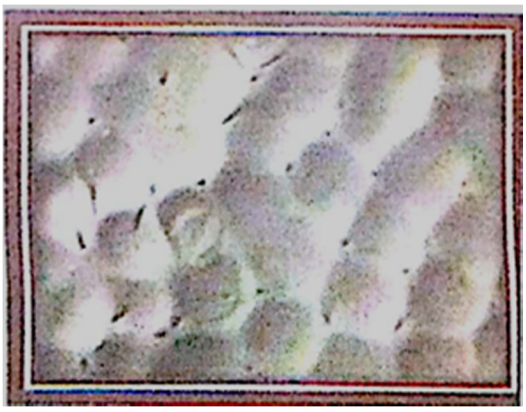
NAIL POLISH IMPRESSION TECHNIQUE OMMATIDIA

NORMAL ORDERED ARRAY



Aβ 42

DONEPZIL TREATED



VO TREATED

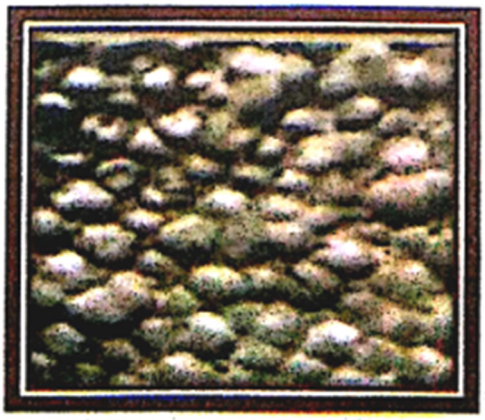
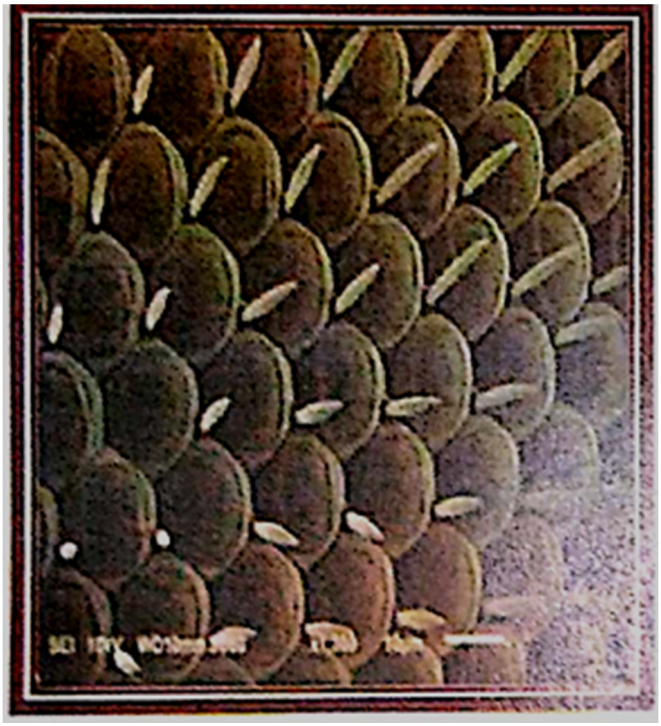
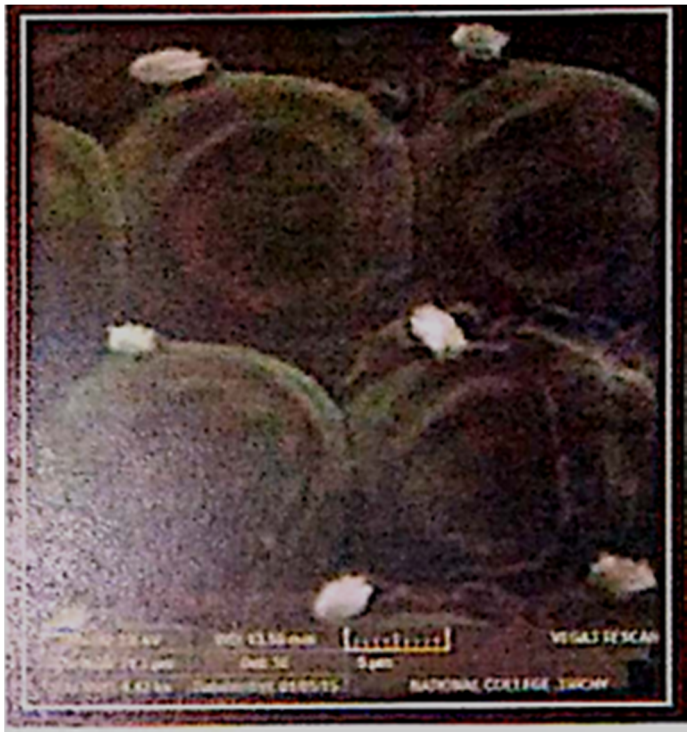


PLATE 16

EVALUATION OF OMMATIDA UNDER SEM

NORMAL ORDER ARRAY





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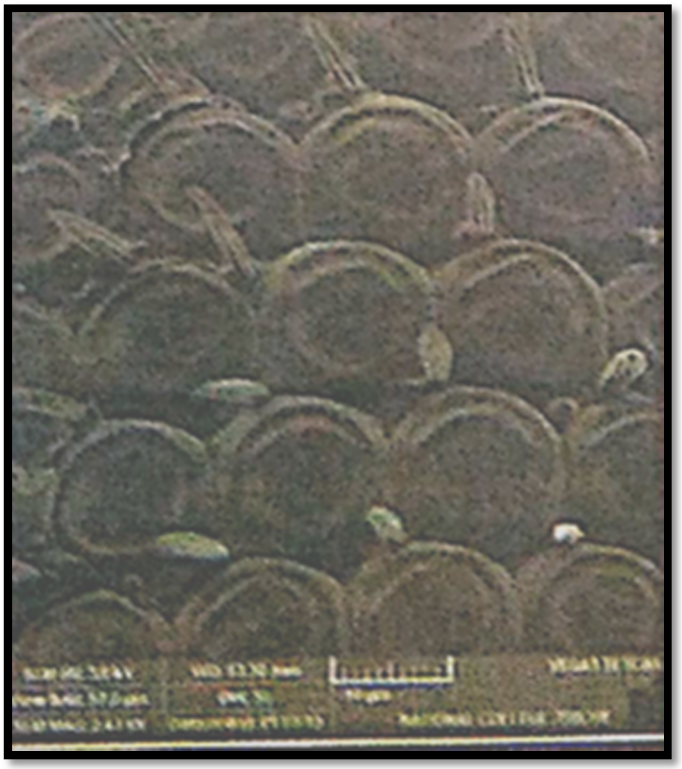
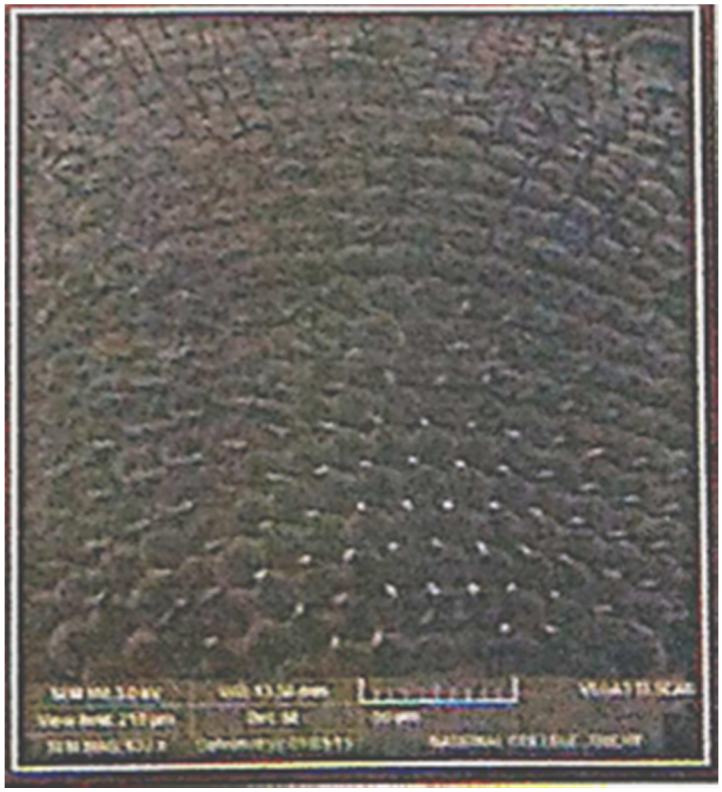


DONEPEZIL TREATED



DISCUSSION

DISCUSSION

The dissertation covers a study on widely available member of the family Myrtaceae known botanically *Syzygium aromaticum* commonly called as ‘clove’ in English and Kirambuin Tamil. The *Syzygium aromaticum* leaves of really do not have any match as a cheap natural and easily available plant. It was reported that Myrtaceae family members have phyto constituents useful in the treatment of various diseases and was also claimed that these plants merit detailed study which can prove useful in the discovery of lead compounds leading to novel and more efficacious drugs. Leaves of *Syzygium aromaticum* of this family, is traditionally known to be useful for the treatment of wide panel of diseases like and as antioxidant, antimicrobial, antinociceptive, antiviral.Various scientific investigation of the leaves showed anti-diabetic activity, anti-microbial activity, antiulcer, analgesic, anti-inflammatory ,anti-microbial, anti-oxidant, hepatoprotective, antihypertensive and wound healing activity.

Clove is known to possess antibacterial properties and is used in various dental creams, tooth pastes, mouth washes, and throat sprays to cleanse bacteria. It is also used to relive pain from sore gums and improves overalldental health. In dentistry, eugenol in combination with zinc oxide isused for temporary filling of cavities.Clove is an anodyne (an agent that soothes or relives pain) for dental emergencies11. Cloves are aphrodisiac (an agent for arousing or increasing sexual desire or potency).Clove is used as an anti-inflammatory agent, due to itshigh content of flavonoids. Aroma therapists use pureclove oil to cure the symptoms of rheumatism andarthritis. Clove is used as a carminative, to increase hydrochloricacid in the stomach and to

improve peristalsis. Apply the paste of clove powder in honey to treat acne. Paste of clove powder in water promotes faster healing of cuts and bites. Cloves can effectively cure many digestive problems. It is having medicinal qualities to cure flatulence, loose motions, indigestion and nausea. Cloves are useful in relieving the symptoms of diarrhoea, gastric irritability and vomiting.

The economic aspect of this crop evidently proved that as commercial crop. In fact the revenue generated by this crop can be further magnified by many folds, if its medicinal applications are scientifically explored well. By a well-coordinated effort, we can properly exploit this plant. Therefore research on development of herbal products from this plant is required to be initiated immediately for exploring the unique potential of this crop which would also minimize the menacing wastage especially the leaves. It may be further envisaged that the revenue generated by this plant would easily exceed that generated by any major crop of the country even with a present level of traditional agro economic practices. Therefore a well-coordinated effort by the farmers, traders, scientist, technologists, extension workers, physician, administrators, and policy makers is required to be initiated to boost up the national economy as well as the proper exploitation of this for proper therapeutic purpose. The review of literature showed some lacuna exists in the pharmacological, phytochemical, and pharmacological studies in the leaves of *S.aromaticum.*

PHARMACOGNOSTICAL STUDIES :(PLATE 1-12)

Morphological and micro morphological examination and characterization of medicinal plants have always been accorded due credentials in the pharmacological studies. There was no detailed pharmacognostical work has been carried out including botanical identity based on micro morphology in this leaves of this plant.

The application of morphological studies in drug analysis is pertinent in the field of crude drug authentication. It was studied for the leaf. Interpretation of the morphological characteristics based on different parameters, for the plant organs give a guideline for the diagnosis of the original plant and its adulterants.

Colour, size, shape, margin, texture, arrangement were observed and compared with previous data.

Microscopic techniques help to magnify the fine structure of minute objects and there by confirm the structural details of the plant drug. Though the microscopical evaluation cannot provide complete profile, still it can offer supporting evidences which when combined with other analytical parameters can be used to obtain full evidence for standardization and evaluation of herbal drugs. Consideration must therefore be given to the types of cells and cell inclusions and the manner in which they are distributed in different organ of the plants. The habit and habitat and the various morphological characters of the various parts have been studied after proper identification and authentication Leaves are simple,opposite,coriaceous,exstipulate,glabrous and aromatic.Size 7.5-12.3 x2.5-3.75 cm, Green in colour with entire margin

and cuneate base.Apex shortly or broadly blunty acuminate and Petiole 2-3 cm long.

T.S of Midrib grooved from above erect downward flat on the adaxial side, And convexity on the adaxial side.It is made up small rectangular thick wall uniseriate cells, covered by a thick cuticle. In surface view polygonal in shape amphistomatic / ranunculaceousfew in upper and more in lower epidermis.

Transverse section of lamina shows an upper epidermis with a cuticle. Stomata are present on both the epidermis followed by two layer of palisade cells containing chloroplasts. The spongy mesophyll is made up of 2 - 4 layers of cells and seen in between the palisade and lower epidermis cells. Some of the cells of the spongy parenchyma contain oil contents as secretary cavities developed initially schizogenous but ultimately lysigenous at maturity with a breakdown of all the secretary cells within the gland lumen. Oil cavities located close to both surfaces, glandular and lined with epithelial like cells. Cells containing tannin are very common. The vascular system consists of a U shaped or almost closed circle of separate collateral vascular bundles. Small vascular bundles are scattered towards the peripheral region. Each vascular bundle is encircled by a single row of bundle sheath. Distinct vein islets and vein terminals(some are forked) were clearly seen.

Scanning electron microscopy (SEM) study showed no diagnostic features and new kind of micro-constituents not previously recognized and apparently simple structure which may be extremely complex were observed.

The plant drugs are generally used in the powdered form where the macro morphology is generally destroyed, so the diagnosis of the plant

through the microscopical character is essential. The powdered crude drugs can be identified based on the presence or absence of different cell types.

In powdered microscopy observed ranunculaceous stomata, wavy walled epidermal cells, spiral annular vessels, phloem cells, sieve tube and companion cells, fibres, scizolysigenous secretary cells, tannin containing cells, parenchyma cells. **(Fig-4)**

Quantitative microscopy includes certain measurements to distinguish some closely related species which are not easily differentiated by general microscopy. The **stomatal number** is the oldest technique but a simple method of diagnosis of fragmentary leaf parts. The **stomatal index** is the percentage of stomata in relation to the epidermal cells. Both are very specific criteria for the identification and characterization of leafy drugs. **Vein islet and vein termination number** are another simple technique for distinguishing fragmentary specimens at specific levels. It is used as the distinguishing character for the leaf of the same species or different one.

Palisade Ratio is another criterion for identification and evaluation of herbal drugs. This value remains constant within a range for a given plant species and is of diagnostic value in differentiating the species. This value does not alter based on geographical variation and differs from species to species and that is why it is a very useful diagnostic feature for characterization and identification of different plant species. (Table 4)

The ash content of the crude drug is generally taken to be the residue remaining after incineration. If usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also involve the inorganic matter added for the purpose of adulteration. There is a considerable

difference within narrow limits in the case of individual drug. Hence ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information related to its adulteration with inorganic matter. The ash or residue yielded by an organic chemical compound is a rule to measure the amount of inorganic matter, which is present as impurity. In most cases the inorganic matter is present in small amounts which are difficult to remove in the purification process and which are not objectionable if only traces are present. Ash values are helpful in determining the quality and purity of the crude drug in especially in powdered from. The **acid insoluble ash** is of more value to detect the earthy matter adhering to the drug. In this way one can obtain evidence of the presence of foreign matter, which likely to occur with root, rhizomes and also in pubescent leaves. **The water soluble ash** is used to detect the presence of matter exhausted by water. Insufficient drying favours spoilage by molds and bacteria and makes possible the enzymatic destruction of active principles (Table -5). **Extractive values** of crude drugs determine the amount of active constituents in a given amount of medicinal plant material when exhausted with solvents. It is employed for that material for which no chemical or biological assay method exist. As mentioned in different official books [Anonymous**.,** 1996**,** Anonymous**.,** 2006, Horborne JB., 1973] the determination of water-soluble and alcohol soluble extractive, is used as means of evaluating crude drugs which are not readily estimated by other means. The extraction of any crude with a particular solvent yields a solution containing different phytoconstituents. The composition of these phytoconstituents in that particular solvent depends upon the nature of the drug and solvent used. The use of single solvent can be the means of

providing preliminary information on the quality of a particular drug sample. The **water soluble extractive** values play an important role for the evaluation of crude drugs. It can be used to indicate poor quality, adulteration with any unwanted material or incorrect processing of the crude drug during the drying, storage etc. The **alcohol soluble extractive** is also indicative for the same purpose as water soluble extractive values (Table -7).

Loss on drying at 105^{o}C is determined as the presence of excess moisture is conductive to the promotion of mold and bacterial growth, and subsequently to deterioration and spoilage of the drug (Table -6)

THE PRELIMINARY PHYTOCHEMICAL STUDIES:

The preliminary phytochemical screening reveals the presence of carbohydrates, proteins and amino acids, flavonoids, terpenoids, tannins, sterols, volatile oil. Fixed oil was found to be absent.(Table -8)

The reaction of drugs in powdered form in ordinary light and with filtered UV light is of importance in several cases by the luminosity in UV light by **fluorescent analysis**. Many flavonoids showed distinctive colours under UV light: Bright yellow (6-hydroxy flavanoids and flavones and some chalcones), dark brown (most flavanol glycosides, dark may be (isoflavones and flavonols). Hence this parameter can also be used as a diagnostic tool for the standardization of herbal drugs for the detection of adulterants in crude drugs (Table-9) (Harborne JB., 1973).

Determination of total flavonoid content was found to be 23.1µg/g .Determination of total phenolic content was found to be 3.7mg/g .

Identification of inorganic minerals of the leaves of S.*aromaticum*byenergy dispersive X-ray spectrometer (EDS)showed the

presence of minerals Carbon (78.45%), Oxygen (21.23), Sodium (0.12%), Silica (0.05%), Chlorine (0.08%), Potassium (0.06%) (Table-10). No special characters were observed in SEM analysis.

Volatile oil was isolated from the leaves (VOSAL) by hydrodistillation method (2.5%v/w). VOSAL is pale yellow, aromatic, pungent and greasy to touch. Soluble In petroleum ether, toluene, chloroform, ethanol and immisible with water. RI is 1.533-1.359Specific Gravity: 1.032-1.067and.Optical Rotation: Is -$0^0$50" to - 1^o53.

The GC-MS analyscis of the isolated V.O indicated the presence of following constituents bycomparing with the instrument library. β-caryophyllene, Globulol α-pinene, α-terpinene, β-bisabolene, Eugenol, Limonene, caryophylene oxide, cadinol, m.Cymene.

Encapsulation of bioactive compounds represents a feasible and efficient approach to modulate drug release, increase the physical stability of the active substances, protect them from the interactions with the environment, decrease their volatility, enhance their bioactivity, reduce toxicity, and A significantly large part of current literature on the encapsulation of EOs deals with micrometric size capsules, which are used for the protection of the active compounds against environmental factors (e.g., oxygen, light, moisture, and pH),to decrease oil volatility and to transform the oil into a powder. Encapsulation in nanometric particles is an alternative for overcoming these problems but additionally, due to the subcellular size, may increase the cellular absorption mechanisms and increasing bio efficacy improve patient compliance and convenience

Preparation of nanospheres of volatile oil of the leaves of *S.aromaticum* (SALVONS):

Encapsulation of VO using 1:1 (w/w) ratio EC/MC as the polymeric shell material was performed, resulted in the formation of milky white aqueous suspension. Analysis of the suspension by SEM revealed the presence of almost spherical particles with a dry size average diameter of 424± 102.6 nm. The process gave an EE of 77.5% with a loading level of 44.2% w/w.

PHARMACOLOGICAL STUDIES:

ACUTE TOXICOLOGICAL STUDY:

In continuation of our efforts to verify the safety of VOSALwe performed Brine shrimp lethality assay (BSLA) using free swimming hatched out *Artemianauplii* which based on the ability to kill laboratory cultured brine shrimp . It was observed that 100% of mortality above 1000ppm .LC_{50} for were about 500ppm in 24hrs. 100% mortality was observed at 3ppm for podophyllotoxin positive control. (Table-11).

In the investigation of effect on mutagenesis of the VOSAL we discussed about the need for performing the eye and wing spot assay along with description about *D. melanogaster* life cycle and reproduction, as a model organism in genetics and its similarity to humans.

The IInd stage larvae were selected for experiment. Formaldehyde (0.5%v/v) selected as standard chemical mutagen. VOSAL in the concentration (10, 20µl/ml) range were fed. After eclosion of the exposed flies from larvae stage, the phenotype changes, i.e., eye, color, wing hair, wing spot, wing shape, changes in the wing length and width, abdomen length and total body length were observed. Standard mutagen formaldehyde (0.5%v/v)

produced visual mutations (eye color become pink from brown) and additionally there were noticeable changes in the wing length and width, but remaining factors unchanged when compared to normal flies. The results are tabulated (Table-12). The results observed were that the VOSAL at all concentration employed showed no significant morphological changes in the *D. melanogaster* on comparing formaldehyde exposed flies. This preliminary assay suggests its antimutagenic effect on *D. melanogaster.*

One of the important avenues in drug discovery that holds tremendous potential is use of model genetic organism such the fruit fly, *D. melanogaster*. This model represent intact living system was complex biological patterns and process can be readily examined. More over the similarity between mode of drug action, behavior and gene response in the *D. melanogaster* and mammalian systems, combined with the power of genetics, have recently made the fly a very attractive system to study fundamental neuro pharmacological processes relevant to human disease. The benefit that the use of model organisms like the fly, offers is speed, HTP and dramatically reduced overall cost that together result in an enhanced rate of drug discovery. It is also worthy to note that currently regulations regarding animal care and use (eg. IACUC) that applied to vertebrate animal research do not apply to *Drosophila* (Nichols.D.C.2006).

In this study we evaluated the neuro protective effect of SALVONS using *Drosophila* AD model. Initially we evaluated the effect of 5 and 20µl/g of the *Drosophila* media on food intake of *Drosophila*. We hypothesize SALVONS may also be effective in protecting neurons against beta-amyloid induced neuronal death and there is a lack of information relating to the *in*

vivo neuroprotective effect. In hope of finding phytoconstituents which could modulate APP cleavage and reduce neurotoxic effect from beta amyloid, the present study aimed to investigate the neuroprotective effects of SALVONS on beta amyloid induced neurodegeneration in *Drosophila*. *Drosophila melanogaster* was recently developed model organism for drug/medicinal plant screening for neurodegenerative diseases. It provides many unique features such as highly stable and fully known genetics highly conserved disease pathways, high throughput and very low comparative cost. Most of the genes implicated in human AD pathogenesis have *Drosophila* homologs, including amyloid precursor protein (APP) γ secretase and tau. But there are some dissimilarities, such as the absence of β-secrtase, which cause a defect in endogenous production of Aβ42. In this investigation the *Drosophila* models that overexpress human Aβ 42 would be used. The neurodegeneration would result in reduced lifespan, reduced locomotor activity and eye degeneration. These pathological phenotypes could be observed within few weeks, much faster than the development of these phenotypes in transgenic mice. Hence application of *Drosophila* as model of AD provides excellent tools for performing drug/herbal screens to identify small molecules and herbal formula that can suppress the toxicity associated with Aβ accumulation.

Both concentration did not affect the food intake of *Drosophila* (data not shown) which ensured no experimental differences were due to the alteration of feeding behavior.

SALVONS prolonged the lifespan and improved locomotor function of Aβ42 *Drosophila*:

For lifespan experiment, Aβ42 *Drosophila* showed a reduction of lifespan by when compared to control. SALVONS treatment significantly improved the survival of *Drosophila*.5µl /g of *Drosophila* media, lifespan were increased comparable to the standard drug donepezil ($p<0.001$).

For the determination of locomotor ability, Aβ42 *Drosophila* showed significant impaired locomotion from age of day 9 onwards. SALVON Streated flies showed an improvement in the locomotor activity from age of days 12 to 23. At day 12, 19, and 23 4µl/g of *Drosophila* media resulted in an improvement in locomotion. ($p<0.001$) when compared with the Aβ42 *Drosophila* without treatment.

RESCUED NEURODEGENERATION IN OMMATIDIA OF AB42 EXPRESSING *Drosophila*:

We investigated the effect of Aβ42 *Drosophila* on degeneration of retinal tissue of, which were mainly neurons. Aβ42 *Drosophila* contained significantly more degenerating rhabdomeres compared to normal. The number of degenerated rhabdomeres was 2 ± 0.458. Those treated with SALVONS had significantly rescued rhabdomeres in each ommatidium with an increase of rhabdomere count per ommatidium for the coc dose dependent by which reflected a preventive effect of SALVONS on neurodegeneration. The preventive effect was compared to donepezil, STD drug 10µmol/g of drosophila media. In which there was an increase of rhabdomeres count per ommatidium than the Aβ42 *Drosophila*.

This shows it is an inexpensive and affordable and can be evaluated further for formulations.

In this present study, we demonstrated protection, rescue and most importantly restoration of the impaired movement activity (i.e climbing capability) *Drosophila melanogaster*, a valid model of AD. It provides a framework for future studies in AD patients. Elucidation of their mechanism of action will provide new insight for new targets.

CONCLUSION

CONCLUSION

The present investigation high lights the pharmacognostical, phytochemical studies of the leaves of *Syzygium aromaticum* family *Myrtaceae*and potential protective action against the beta amyloid induced Alzheimer disease in the *Drosophila* model and found more effective therapeutically than the synthetic counterpart without toxicity of its VOSAL. It is commonly called as *clove* widely cultivated and easily available plant. Ethnomedical information revealed that it is used in various ailments for long time all over the world. The tremendous economic potentiality of this cash crop remains neglected by the scientists, technologists, physician, traders, administrators, policy makers, farmers etc.

The morphological evaluation showed the adherence of general characters to the family.

Detailed microscopical characters of the leaves showed the presence of usual leaf tissue arrangements along with syzolysegenous secretary cavities, ranaunculaceous stomata, two layers of palisade cells, U shaped bicollateralvasucular bundle and tanniniferous cells in ground tissue. Petiole microscopicalstructure showed U shaped vascular bundle and secretary cavities.

Scanning Electron Microscopic (SEM) observation SEM study of leaf provides detailed surface information. Adaxial and abaxial epidermal surface is smooth with no ornamentation. Quantitative parameters, Vein islet and termination numbers, stomatal number, stomatal index, palisade ratio, loss on drying, ash values, extractive values were determined and presented. Preliminary phytochemical screening showed the presence of carbohydrates,

proteins and amino acids, alkaloids, flavonoids, saponins, tannins, terpenoids, phytosterol, mucilage, volatile oil and absence of fixed oil.

Quantitative inorganic elemental analysis by Energy dispersive X-Ray analysis (EDS) showed the presence of Carbon (78.45%), Oxygen (21.23), Sodium (0.12%), Silica (0.05%), Chlorine (0.08%), Potassium (0.06%). The pharmacologically important phytoconstituents polyphenols as total phenolic content and flavonoids were determined.

The VOSAL from the leaves was isolated and its GC-MS profile was presented. Presence of Eugenol (85.2%), Eugenal acetate 0.37%, β-caryophylline 9%, α- humulene, vindiflorine, β- Phellondrene, m-cymene, limonene.

Nanospheres of volatile oil of the leaves of *S.aromaticum* (SALVONS) was prepared. Encapsulation of VO using 1:1 (w/w) ratio EC/MC as the polymeric shell material was performed, resulted in the formation of milky white aqueous suspension. Analysis of the suspension by SEM revealed the presence of almost spherical particles with a dry size average diameter of 424± 102.6 nm. The process gave an EE of 77.5% with a loading level of 44.2% w/w.

The **3R's** ethical principle (**R**eduction, **R**efinement and **R**eplacement) was implemented that help to minimize harms to vertebrate animals used in science.

Acute toxicity study was performed by bench top bioassay BSLA (Brine Shrimp Lethality Assay) LC_{50} of VOSAL was found to be 500ppm and are non-toxic. (Standard toxin podophyllotoxin showed 100% mortality at 3ppm itself.) Effect of VOSAL on mutagenesis *in vivo* was investigated first time for

using *Drosophila* model organism to fill up the lacuna and found no effect on mutagenesis.

In our study we used valid model genetic organism *Drosophila melanogaster* as a system biology research approach since it places specific molecular targets within a context of overall biochemical reaction. The similarity between mode of drug action, behavior, and gene response in model organisms and mammalian systems, combined with the power of genetics made them very attractive system to study fundamental neurophamacological processes relevant to human diseases especially the Alzheimer, a common progressive neurodegenerative disorder for which at present no causal treatment is available . We investigated the *in vivo* neuroprotection of the SALVONS on the representative of Alzheimer disease model of transgenic *Drosophila* by overexpression of beta amyloid protein, Aβ42 .In the present study we have presented the first evidence that the SALVONS could significantly ameliorate the adverse morphological changes from Aβ42 protein in *Drosophila,* as indicated by prolonging the lifespan, by improving locomotor abilities and rescuing neurodegeneration in ommatidia Aβ expressing *Drosophila* which is comparable with donepezil. So it demonstrated the novel use of SALVONS against Aβ induced neurodegeneration which may be mediated through the multiple components of the VOSAL. Based on this findings we suggest developing VOSAL as a potential therapeutic intervention for neurodegenerative diseases like Alzheimer's disease. VOSAL effectively protects, rescue and most importantly restore the impaired movement activity (i.e climbing capability) in *Drosophila melanogaster*, a valid model of AD. It provides a framework for future studies

to assess to suppress oxidative stress and to restore and or maintain locomotor activity in AD patients. Elucidation of their mechanism of action will provide new insight for new targets.

In conclusion, the leaves of *S.aromaticum* may be further investigated for the development as novel non toxic preventive/treatment interventions for life threatening neurodegenerative diseases such as AD. But to confirm our findings further investigations of these effects to mammalian model is necessary. Further pharmacokinetic studies are also required to understand the post metabolism ingredients along with the clinical efficacy and safety in human.

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